PLASMINOGEN ACTIVATOR INHIBITOR-1, DIABETES, AND VASCULAR DISEASE

by

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A thesis submitted to the University of Ottawa Faculty of Medicine in partial fulfillment of the requirements for

the degree of Doctorate in Philosophy, Cellular and Molecular Medicine

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Abstract

Patients with established coronary artery disease (CAD) remain at elevated risk of major adverse cardiac events (MACE). Specifically, stented coronary artery remains the highest-risk coronary lesion with annualized adverse event rates as high as 8-12% in the following year largely due to in-stent restenosis (ISR) and stent thrombosis. Plasminogen activator inhibitor-1 (PAI-1), an anti-fibrinolytic protein, has previously been associated with CAD with known mechanism of action to regulate the pathophysiological changes associated with in-stent restenosis and stent thrombosis. Moreover, extracellular vesicles (EVs) originating from circulating blood and vascular cells are increasingly being utilized as biomarkers and mediators of vascular disease. We first demonstrate the analytical and biochemical performance of plasma PAI-1 in patients with established CAD. Specifically, PAI-1 performs similarly to established biomarkers including C-reactive protein and NT-proBNP with an analytical (CV_a = 4.1%), intra-individual (CV_i = 44.0%), and inter-individual (CV_g = 118.6%) coefficients of variation. Following this, we demonstrate that plasma PAI-1 is not associated with MACE in one-year follow-up, but reduced levels of PAI-1 remain associated with unplanned revascularization. Subsequently, we sought to evaluate the relationship between PAI-1 and EVs in humans with platelets being a common source of origin. In the largest study of EV to-date in CAD (n=489), we demonstrate the strong predictive ability of PAI-1 platelet-derived EVs (PAI-1+ PEV) with MACE following revascularization. Patients with high circulating levels of PAI-1^+ PEV had higher rates of MACE (262.3 vs. 103.0 events per 1,000 person-years; hazard ratio (HR) 2.19; 95% CI, 1.07-4.52; and HR 2.67; 95% CI, 1.22-5.84, discovery and validation cohorts, respectively). Furthermore, we reveal that high PAI-1+ PEV fractions did not enhance thrombogenicity but promoted a pro-inflammatory vascular smooth muscle cell (VSMC) state by enhancing proliferation and migration, through up-regulation of pro-inflammatory genes such as KLF4. Inhibition of the PAI-1-LRP-1 interaction by TM5275 dampened the pro-inflammatory VSMC response, whereas inhibition of the PAI-1-vitronectin interaction by tiplaxtinin had no such effect. Our data reveals the potential of PAI-1+ PEV as a biomarker in the post-revascularization population and postulates the mechanism in an *in vitro* model of VSMCs.

Accordingly, our data demonstrates the potential of PAI-1 PEV as a strong biomarker following revascularization and PAI-1 inhibition by TM5275 is a promising strategy to reduce the pro-inflammatory VSMC state associated with ISR.

Acknowledgements

I am deeply grateful for the following individuals (trainees and mentors) for all their contributions and mentorship with my graduate and medical training, for this would not have been possible without their time and dedication.

To the members of the Hibbert lab, Dr. Trevor Simard, Dr. F. Daniel Ramirez, and Dr. Pietro Di Santo, your guidance and continued mentorship have shaped my research career and provide continued aspiration for a clinician-scientist career. Specifically, Dr. Simard, you have taught me the work-ethic required in order to be a successful and passionate clinician-scientist.

The members of the Thesis Advisory Committee, Dr. David Allan, Dr. Wenbin Liang, and Dr. Alexander Sorisky, thank you for the guidance in leading the direction of the project over the last few years.

To all the other trainees who supported and continue to support our projects, thank you. This includes Alisha Labinaz, Taylor Gillmore, Shan Dhaliwal, Cameron Stotts, Stephanie Skanes, Cole Clifford, Dr. Denuja Karunakaran, Dr. Adil Rasheed, Dr. Anne-Claire Duchez, Dr. Simon Parlow, Dr. Dylan Perry-Nguyen, Dr. Pouya Motazedian, Dr. Graeme Prosperi-Porta, Dr. Annie Hung, Dr. Joanne Joseph, Dr. Omar Abdel-Razek, Dr. Kiran Sarathy, Dr. Jeffrey Marbach, and Dr. Robert Moreland. Your passion and dedication remain the backbone to all the research we are able to complete.

To our collaborators, Dr. Katey Rayner, Dr. Fengxia Xiao, Dr. Suresh Gadde, Dr. Dylan Burger, and Dr. Marie Lordkipanidze, thank you for your support. Your expertise and guidance were valuable in order to elucidate the mechanistic role of PAI-1, especially in the last chapter of the thesis.

All this work was supported by the Vanier Canadian Institutes of Health Research (CIHR) Canada Graduate Scholarship. Moreover, this work by Dr. Hibbert's laboratory was supported by the Innovation Fund of the Alternative Funding Plan for the Academic Health Sciences Centres of Ontario and Canadian Foundation of Innovation.

I would like to thank my family and friends for all their support over the years, especially during the COVID-19 pandemic. None of these accomplishments would be possible without you. I especially would like to thank Katlyn Taylor for her patience, guidance, and support during these years.

I forever owe my deepest gratitude to my supervisor, Dr. Benjamin Hibbert. Your guidance and mentorship even during our first meeting truly shaped my career aspirations. Your continued support and mentorship over the past four years allowed the completion of this work. Thank you and one day I aspire to be even a fraction of the clinician-scientist and mentor you are.

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List of Abbreviations

AUC – area under the curve

CAD – coronary artery disease

CRP – C-reactive protein

DAPT – dual antiplatelet therapy

DM – diabetes mellitus

ELISA – enzyme linked immunosorbent assay

EV – extracellular vesicles

FITC – fluorescein isothiocyanate

FSC – forward scatter

HbA1c – hemoglobin A1c

HR – hazard ratio

IQR – interquartile range

LRP1 – low-density lipoprotein related receptor-1

MACE – major adverse cardiac events

MFI – median fluorescence intensity

MI – myocardial infarction

NT-proBNP – N-terminal pro b-type natriuretic peptide

PAI-1 – plasminogen activator inhibitor-1

PAI-1$^+$ PEV - plasminogen activator inhibitor-1 positive platelet-derived extracellular vesicles

PBS – phosphate buffered saline

PCI – percutaneous coronary intervention

PE - phycoerythrin

PEV – platelet extracellular vesicles

ROC – receiver operating characteristic

SSC – side scatter

tPA – tissue-type plasminogen activator

uPA – urokinase-type plasminogen activator

VSMC – vascular smooth muscle cells

Chapter 1

Literature Review of the Role of Plasminogen Activator Inhibitor-1 in Coronary Pathophysiology

1.1 Preface

This chapter has been previously published as a review article in *Thrombosis Research*:

> **Jung RG**, Simard T, Labinaz A, Ramirez FD, Di Santo P, Motazedian P, Rochman R, Gaudet C, Faraz MA, Beanlands RSB, Hibbert B. Role of plasminogen activator inhibitor-1 in coronary pathophysiology. *Thromb Res*. 2018;164:54-62

1.2 Abstract

The standard of care for obstructive atherosclerotic coronary disease is revascularization, predominantly achieved via percutaneous placement of a stent with concurrent medical therapy. Advancements in percutaneous coronary intervention (PCI) have dramatically improved outcomes. However, major complications from PCI due to target lesion failure continue to occur at rates between 5-10% in the first twelve months following intervention limiting its therapeutic efficacy. Plasminogen activator inhibitor-1 (PAI-1) is a protein of interest for both arterial remodelling and thrombotic risk as it regulates cell migration and vascular thrombosis. Elevated PAI-1 antigen levels have been identified as a potential biomarker for coronary artery disease and metabolic syndrome while being modulated by a number of atherosclerotic risk factors. Although linked by some studies as a marker of disease severity and prognosis, it remains to be understood whether it is also a mediator and/or therapeutic target of vascular disease. In this review, we discuss the current understanding of PAI-1 in vascular disease and its potential role in in-stent restenosis and stent thrombosis.

1.3 Introduction

Cardiovascular diseases remain the leading cause of morbidity and mortality in North America. Obstructive coronary atherosclerotic disease can present in both stable and acute forms and is commonly treated with a combination of medical therapy and coronary revascularization by percutaneous coronary intervention[1]. Atherosclerotic plaque rupture or vascular injury sustained during stent implantation, can initiate the coagulation cascade leading to pathogenic clot formation in the blood vessel. *In situ* thrombosis which occurs after plaque rupture or stent implantation can then lead to ischemia, myocardial cell necrosis and myocardial infarction (MI)[1].

The hemostatic cascade maintains a dynamic homeostasis between blood remaining in its free-flowing state while still allowing rapid response to repair vascular injury with fibrin clot formation, thrombus formation disturbs this hemostatic balance [2]. Virchow's triad for thrombogenesis stipulates that vessel wall changes including endothelial dysfunction, alterations in blood flow (ie. blood stasis), and abnormal blood constituents are required for thrombus formation [3]. In myocardial infarction, thrombosis occurs in the presence of significant vascular injury in the form of plaque rupture and disturbance in blood flow. Thrombus formation may initially be triggered by platelet activation which occurs due to stimulation by sub-endothelial matrix agonists such as von Willebrand factor, collagen, and fibronectin exposed during vascular injury [4]. Subsequently, active platelets provide a nidus for procoagulant proteins resulting in fibrin polymerization to complete thrombus formation. Thrombi contain circulating erythrocytes, leukocytes, and platelets, in addition to factor XIIIa which cross-links fibrin to increase its stability [5].

To counter thrombus generation, fibrinolysis is activated by the conversion of inactive plasminogen to plasmin on thrombus or cell surfaces by serine proteases such as tissue or urokinase plasminogen activator (t-PA and u-PA, respectively) [6]. Thrombus surfaces are key sites of action as t-PA complexed to fibrin demonstrates a 500-fold increase in the rate of activation of plasminogen [7]. The end-product of serine protease action is plasmin which cleaves lysyl- and arginyl-peptide bonds of fibrin into fibrin degradation products to resolve the thrombus [8]. Thus, development of thrombus depends on the nidus or initiating signal

and the balance of pro and anti-thrombotic pathways at the level of the injury, which is highly regulated to ensure balance in hemostasis.

Plasminogen activator inhibitor-1 (PAI-1) is an important inhibitor of the fibrinolytic pathway. Under normal physiological conditions, PAI-1 inhibits t-PA and u-PA by forming a 1:1 stoichiometric complex, preventing plasmin formation. However, elevated levels of PAI-1 have been associated with various pathologic conditions including myocardial infarction, diabetes, and metabolic syndrome - potentially contributing to the observed increased rates of adverse events [9-11].

Thus, the current review seeks to summarize the current knowledge of the biology of PAI-1 and potential contribution to atherothrombotic events and development of vascular lesions. Specifically, a focus will be made on translational applicability of PAI-1 in diagnosis, treatment, and ultimately, prevention of vascular diseases.

1.4 PAI-1 Structure and Function

1.4.1 PAI-1 Gene

PAI-1 is a member of the serine protease inhibitor (serpin) family. Serpins are homologous proteins that arose from a common ancestral gene 600 million years ago [12]. About 40 different members of the serpin family exist in viruses, plants, and higher organisms [8]. Other members of the family include PAI-2 (Serpin B2), PAI-3 (Protein C inhibitor or Serpin A5), and anti-thrombin [11]. The PAI-1 gene is found on chromosome 7q21.3-q22, spans 12.3kb, and comprises 9 exons and 8 introns [11, 13, 14]. The gene codes for two distinct mRNA transcripts that vary in length (2.3 and 3.2 kb) at the 3' end due to alternative polyadenylation [15]. The longer transcript appears to contain an AT rich sequence that confers greater mRNA stability [15].

The 5' promoter region of the PAI-1 gene contains numerous regulatory elements and its potential role in cardiovascular diseases has been characterized in detail (Error! Reference source not found.)[11, 14, 16]. F or example, the PAI-1 promoter region contains a glucocorticoid-response element (GRE) as well as an aldosterone response site that upregulate expression of PAI-1[17, 18]. These receptor elements have potential

implications in hypertension. Indeed, angiotensin II stimulates PAI-1 expression and release in a dose- and time-dependent manner [19]. Moreover, it appears that glucocorticoids such as dexamethasone and aldosterone enhance the effect of angiotensin II on PAI-1 antigen production [20]. Notably, a VLDL response element is located at -672 to -657 in the promoter of PAI-1 gene which provides a positive interaction between PAI-1 and VLDL [21]. This has potential clinical implications as elevated plasma triglyceride levels such as VLDL is observed in both diabetes [22] and metabolic syndrome [23]. The PAI-1 promoter also contains two Sp1 sites between -85 and -42 upstream of the transcription initiation site that is regulated positively by glucose and glucosamine in a time- and concentration-dependent manner [24]. This positive association with elevated glucose reveals a potential consequence in diabetes, where a dysregulated level of glucose and insulin resistance contribute to elevated PAI-1 levels [25]. Finally, Smad binding element (SBE) and p53 responsive element (p53RE) exist at positions -740 to -647 and -167 to -125 in the PAI-1 promoter, respectively, potentially playing a role in various cancers [26, 27]. These conserved elements shed light on important regulatory mechanisms which may impact PAI-1 levels and function *in vivo* in clinical conditions.

1.4.2 PAI-1 Polymorphism (-675 4G/5G)

A well-studied single nucleotide insertion/deletion (4G/5G) polymorphism exists in the promoter of PAI-1 (position -675) characterized by 4-5 guanine nucleotides. Both polymorphs bind a transcriptional activator, but the 5G allele can also bind a transcriptional repressor protein [28]. This mutation is associated with regulation of basal and inducible expression of PAI-1 [29] and in the 4G/4G genotype, associated with higher PAI-1 antigen levels, exists a modest increase in MI risk (OR 1.20, 95% CI: 1.04, 1.39, p =0.04) [28, 30]. However, in humans, this polymorphism relationship with PAI-1 expression is confounded by elevated BMI [31], insulin resistance [32], hypertension [33], dyslipidemia [34], diabetes [35], smoking [36]. In addition, PAI-1 is an acute phase reactant in response to inflammatory cytokines such as IL-1, TNFα, and hypoxia [37]. A Mendelian randomization meta-analysis was performed accounting for components of metabolic syndrome

demonstrating an increased MI risk in those carrying PAI-1 4G variant (OR 1.09, 95% CI: 1.01, 1.18) [38]. In addition, 4G carriers had higher PAI-1 activity than 5G carriers by 1.1 IU/mL (95% CI: 0.7, 1.5) and faced an elevated MI risk [38]. Finally, Nikolopoulos et al. presented a meta-regression model which revealed an association between the risk of MI conferred by PAI-1 4G allele, cholesterol and triglyceride levels, and PAI-1 activity levels. Accordingly, multiple confounding variables lead to variation in PAI-1 antigen and activity levels in humans independent of polymorphisms, greatly limiting the applicability of the 4G/5G polymorphism as a biomarker [39].

1.4.3 PAI-1 Protein Structure

The PAI-1 gene codes for a 379 or 381 amino acid single-chain glycoprotein and a 23 amino acid signal peptide for secretion [40]. It has a molecular weight of 50 kDa and a pI of 4.5-5.0 [41]. PAI-1 has been studied through X-ray crystallography, which allowed the elucidation of components of PAI-1[42]. Specifically, PAI-1 has a tertiary structure consisting of three β-sheets (A to C), nine α-helices (A to I), and a reactive centre loop (RCL) consisting of 26 amino acids labelled as P16 to P10' with a bait peptide bond (P1-P1') that mimics the substrate of serine proteases [43, 44].

The reactive centre (P1-P1') of PAI-1 (Arg346-Met347) acts as the pseudo-substrate residues within the RCL that is targeted by serine proteases [11, 44]. Inhibitory serpins such as PAI-1 are "suicide inhibitors" which inhibit serine proteases such as t-PA and u-PA by forming a 1:1 equimolar complex through the interaction of the RCL with the active site of the serine protease [44]. The protease then cleaves the RCL forming an acyl-enzyme intermediate and the next step occurs with two possibilities: PAI-1 can undergo a transition to trap the protease in the inactive complex [45] completing the suicide pathway or proteases cleave the RCL of PAI-1 and prematurely release the protease leaving PAI-1 in the inactive form while liberating the active serine protease requiring another PAI-1[42, 46]. PAI-1 is the primary endogenous inhibitor of t-PA and u-PA with second-order kinetic rate constants ranging between 0.45–2.7x10^{-7} /M·s [47]. PAI-1 is promiscuous in that it also inhibits plasmin and trypsin with second-order rate around 10^{-6} /M·s [11].

It exists in several conformational states within the human body [9] with its half-life increasing in lower pH settings such as in ischemic tissues [37].

1.4.4 PAI-1 Synthesis

PAI-1 is synthesized and secreted by vascular endothelial cells, adipocytes, hepatocytes, cardiomyocytes, fibroblasts and platelets [48, 49]. In humans, plasma levels of PAI-1 exceed >4:1 ratio with t-PA, and the majority of PAI-1 is cleared by the liver with a half-life of 5 minutes [9]. PAI-1 is synthesized in its active form but is spontaneously converted to its latent state with a half-life of 2 to 3 hours [50, 51]. PAI-1 antigen levels are found in low levels in human plasma, ranging anywhere between 5-30 ng/mL in healthy individuals [9, 52]. In human tissues and blood, a fraction of PAI-1 antigen is found in the active form with activity levels (IU/mL) representing between 30-100% of the antigen levels [53].

PAI-1 antigen measurements capture the entire PAI-1 content in the form of free and active PAI-1 (or PAI-1 activity), PAI-1 complexed with t-PA or u-PA, and latent PAI-1. Although PAI-1 antigen and activity levels appear to be correlated [54], antigen levels do not necessarily reflect PAI-1 activity. First, complex formation between PAI-1 and t-PA or u-PA would reduce PAI-1 activity levels [55]. PAI-1 activity is influenced by techniques used during PAI-1 isolation such as freeze-thaw cycles or sonication [39]. In addition, low temperature, high salt concentrations, and low pH during isolation influences the half-life of active to latent PAI-1 conversion, thereby reducing PAI-1 activity levels [56].

Another reason why PAI-1 activity does not reflect the antigen level is due to other proteins such as vitronectin. Vitronectin is present in the plasma, extracellular matrix, megakaryocytes, and α-granules of platelets, which stabilizes PAI-1 by binding to residues 101-123 [57-59]. Vitronectin was demonstrated to extend the half-life of active PAI-1 and is concentrated at sites of injury or remodeling [60] promoting thrombosis and neointima formation [61].

1.5 Platelet PAI-1

1.5.1 Platelet-derived PAI-1

When vascular injury occurs, active platelets release their α-granular content [62]. Initially, α-granule release was thought to be uniquely associated with hemostasis and thrombosis, but has since been linked with inflammation, angiogenesis, malignancy, and atherosclerosis [63]. One major protein of interest from this α-granule milieu is PAI-1. Thrombi formed within arteries contain 2-3 times more platelets than their counterparts formed within veins. In addition, PAI-1 antigen levels were found up to be 30 times greater within a thrombus than in the vascular milieu, the source of which is believed to be platelet-derived [64]. In addition, Faye *et al.* observed PAI-1 in coronary artery thrombi in pigs and found its activity to be >2000 times that of plasma PAI-1 [65]. Although human platelet PAI-1 activity is reduced compared to porcine, this experiment suggests that active PAI-1 concentrated at the site of thrombus formation may be an important factor of fibrinolysis resistance by inhibiting t-PA. Other than PAI-1, platelets contain other proteins involved in the coagulation cascade which impacting clot lysis including α2-antiplasmin, a serpin which inhibits plasmin, and factor XIII which cross-links fibrin to create a more resistant thrombus [66].

1.5.2 Regulation of Platelet PAI-1

Human platelets contain PAI-1 mRNA [67], miRNAs, and ribosomes that are actively involved in translation of PAI-1 antigen. Contrary to the common belief that PAI-1 was synthesized only in megakaryocytes, Brogren *et al.* demonstrated that platelets regularly synthesize active PAI-1 and *in vitro* synthesis rates were about 35x greater than steady-state plasma levels of PAI-1 [68]. In fact, Brogren *et al.* verified the existence of large quantities of PAI-1 mRNA within platelets and active PAI-1 synthesis with a translation rate that is further enhanced after thrombin-induced activation of platelets [68]. In addition, platelets contain miRNAs which can complex with ribonucleoprotein complexes (miRNP) including Argonaute (Ago) proteins to cause translational suppression [69]. Moreover, thrombin induced activation of

PAI-1 can be explained by the dissociation of Ago2 to PAI-1 mRNA, suggesting a role for miRNA regulation of platelet protein expression [70].

An additional level of translational regulation in the 3'UTR of PAI-1 mRNA is observed in platelets through the AU binding proteins T-cell-restricted intracellular antigen-1 (TIA-1) and Human antigen R (HuR)(Error! Reference source not found.)[71]. HuR, an mRNA stabilizing protein, upon platelet activation r emained strongly associated with SERPINE1 mRNA. However, TIA-1, a known translational repressor became dissociated with SERPINE1 upon platelet activation[70]. Theoretically, Ago2, HuR, and TIA-1 may form protein complexes on the mRNA 3'UTR as they bind different sites within the region. Although a mechanistically unique form of PAI-1 regulation exists in platelets, it is rather unlikely that changes in platelet activation contributes to *de novo* synthesis of PAI-1. However, biologically important synthesis following activation has not been excluded [72].

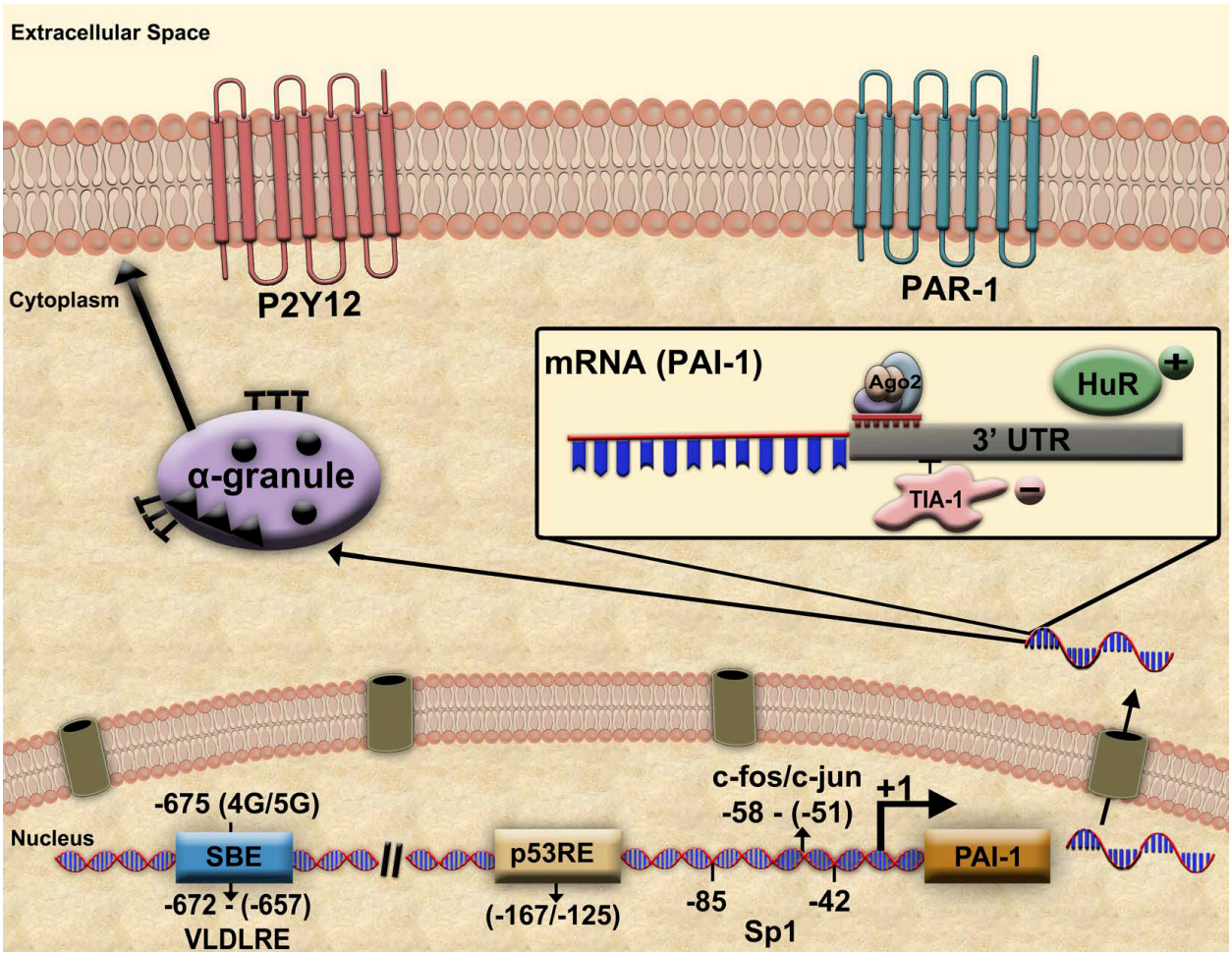


Figure 1-1. Plasminogen activator inhibitor-1 (PAI-1) transcription and translation regulation in megakaryocytes.

(A) The promoter region of PAI-1 is regulated by the VLDL response element (VLDLRE) located between -672 and -657 BP. In addition, PAI-1 polymorph site exists at position -675, where 4G upregulates PAI-1 transcription whereas 5G downregulates PAI-1 transcription. Positions -85 and -42 contain two Sp1 sites which are positively regulated by glucose and glucosamine. In addition, positions -58 to -51 is bound by the c-fos/c-jun heterodimers or AP-1 which are regulated by oncostatin M and IL-1. Finally, at an epigenetic level of regulation, PAI-1 contains a Smad binding element (SBE) and p53 responsive element (p53RE) at positions -740 to -647 and -167 to -125, respectively; upon which a complex formation positively regulates PAI-1 expression. (B) PAI-1 mRNA is regulated at the 3' untranslated region (3' UTR) by microRNA ribonucleoprotein complexes (miRNP) which includes the argonaute (Ago) proteins, most predominantly, Ago2 in platelets. Indeed, thrombin can induce PAI-1 synthesis through the dissociation of Ago2-miRNP complex. In addition, T-cell restricted intracellular antigen-1 (TIA-1) and Human antigen R (HuR) bind to the 3' UTR to further regulate PAI-1 expression. HuR is an mRNA stabilizing protein which remains associated during platelet activation; whereas TIA-1 is a translational repressor that becomes dissociated with PAI-1 upon platelet activation.

1.5.3 Impact of PAI-1 on Cell Migration

Although PAI-1 has a primary role to regulate fibrinolysis, its role in regulation of cell migration has been controversial. PAI-1 appears to either promote or inhibit cell migration depending on the model and cell type studied and contributes to vascular remodeling and neointima formation (Error! Reference s ource not found.)[73]. The three potential pathways in which PAI-1 paradoxically regulates cell migration will be discussed.

Vitronectin has been shown to promote neointima formation in smooth muscle cells [61] through its interaction with integrin and uPAR receptors[74]. Recent evidence has shown that PAI-1 directly stimulates vitronectin expression by binding to the low-density lipoprotein receptor-related protein 1 (LRP1) in smooth muscle cells promoting cell migration[61]. PAI-1 ·vitronectin complex appears to inhibit cell migration and adhesion as PAI-1 binds to vitronectin with a greater affinity at the integrin and uPAR docking site [75].

However, conflicting data has also shown that any conformation of PAI-1 (active, latent, or cleaved) can promote migration by functioning as a signaling molecule and binding to LRP1 through the JAK/STAT signaling pathway in smooth muscle cells[76]. Binding of PAI-1 to LRP1 appears to affect cell proliferation and migration, and further exploration of PAI-1 binding to LRP1 in mouse embryonic fibroblasts (MEFs) reveal findings contrary to previous understanding of JAK/STAT regulation. Instead, an elevation of phosphor-ERK1/2 and β-catenin to promote proliferation and migration has been observed [77]. The authors argue that different cell lines were used to study the role of PAI-1 in cell migration, which reflect the conflicting signaling pathways regulated by PAI-1 and LRP1 interaction.

For effective cell migration, cell adhesion and detachment is required in the proper extracellular matrix milieu. Indeed, targeting active integrins can result in cell detachment by reduced binding to vitronectin, fibronectin, and collagen[75]. Moreover, low levels of PAI-1 can change the cellular actin cytoskeletal architecture consequently leading to cell motility[76]. PAI-1 appears to regulate cell migration by having a direct effect on extracellular matrix proteolysis. PAI-1 appears to reduce cell migration by

binding to vitronectin in the extracellular matrix promoting proteolytic inhibition by allowing an enhanced binding to u-PA. This will promote an extracellular environment that does not favour cell motility [78].

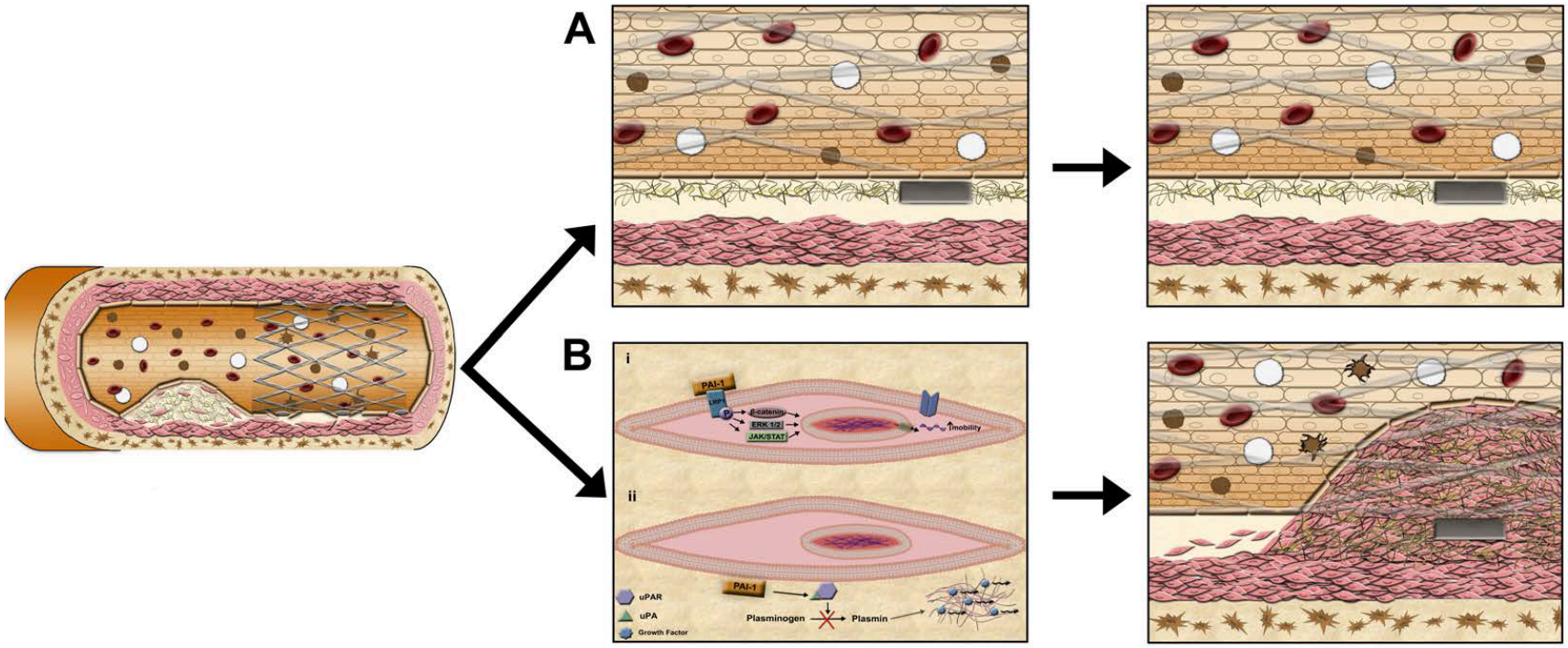


Figure 1-2. In-stent restenosis, a major complication of percutaneous coronary intervention.

(A) Normal re-endothelialization after stent placement signifying optimal coronary repair. (B) In-stent restenosis (ISR), which occurs as a result of neointima formation, is due to the "response-to-injury" paradigm where disrupted endothelium releases cytokines and growth factors to recruit smooth muscle cells (SMCs). Two theories exist for the involvement of PAI-1: (i) PAI-1 acts as an activator of SMC migration by binding to the LDL related-receptor protein 1 (LRP1) and activates the JAK/STAT or the $\boldsymbol{\beta}$-catenin pathway to lead to the production of proteins favoring cell mobility; (ii) PAI-1 directly alters the extracellular matrix composition by inhibiting the natural proteolytic cascade by directly complexing with uPA and has been associated with cell infiltration in atherosclerosis

1.6 Clinical Applications of PAI-1

1.6.1 Performance of PAI-1 as a Biomarker

Elevated plasma PAI-1 levels have been associated with impaired fibrinolytic activity in thrombotic events such as myocardial infarction and stroke [79]. However, in adjusted analyses the association has not been independent of other cardiovascular risk factors [80]. For example, plasma PAI-1 levels are elevated in patients with type 2 diabetes [81], hyperinsulinemia [82], and those with insulin resistance [83]. Elevated PAI-1 activity is associated with metabolic syndrome (i.e. high BMI, waist circumference, and insulin resistance), higher total HDL ratio, younger age, and shorter duration of diabetes [84]. In a recently published systematic review, it was found that patients in the highest PAI-1 quantile were associated with greater coronary artery disease compared to the lowest quantile (OR = 2.17; 95% CI, 1.53 to 3.07) [25]. The authors of this study also identified a potential causal relationship between PAI-1 levels and diabetes[25]. Similarly, in a recent study performed by our group, we identified elevated plasma PAI-1 antigen levels to be predictive of major adverse cardiac events in both primary and secondary prevention populations. Interestingly, in the post-percutaneous intervention population we identified that reduced PAI-1 antigen and activity levels was associated with increased restenosis risk. As PAI-1 is confounded by multiple risk factors, well-designed prospective studies are required to determine if PAI-1 is indeed only a biomarker or if it is indeed a bona fide therapeutic target.

Studies of PAI-1 as a biomarker contain multiple limitations to take into consideration which greatly influences results. First, due to the association of PAI-1 with various risk factors, adequate sample size must be present to adjust for known confounding variables. Second, sampling must consider pre-analytical factors such as diurnal variation and thus must account for time of day given a PAI-1 antigen level difference of 1.24 ng/mL secondary to basal circadian variation in the morning compared to afternoon draws [85]. Most observational studies which measured PAI-1 neglected to report and account for the large diurnal variation observed in PAI-1 antigen and activity levels demonstrated in previous studies. Other pre-analytical factors which influences plasma PAI-1 antigen or activity levels include smoking [36, 86], maximal

exercise [87], high-fat diet [88], intra- and inter-assay variability in PAI-1 antigen and activity measurements [89], and reagents and equipment utilized during PAI-1 isolation from cells. In fact, reagents chosen during isolation can artificially influence PAI-1 activity levels as detergents such as Triton X-100 have been shown to decrease PAI-1 half-life to less than a minute at 37°C [90, 91], and reagents such as urea, SDS, and guanidine-HCl have caused reactivation of PAI-1 [92]. Finally, platelet count and centrifugation load to isolate platelet-poor plasma greatly influences PAI-1 levels [93, 94]. Future studies would be better directed at establishing a standardized sampling method for extracting, isolating, and measuring plasma PAI-1 antigen or activity levels and stratifying patients based on presenting risk factors and time of blood collection.

1.6.2 PAI-1 and Obesity, Insulin Resistance, and Diabetes

Obesity is associated with a number of metabolic derangements and vascular risk factors which contributes to elevated levels of PAI-1 in this population [95]. Not surprisingly, it has been hypothesized that elevated PAI-1 levels may contribute to increased atherothrombotic events observed in obese populations [96]. In contrast to increased platelet production and secretion, PAI-1 in obesity may originate from adipocytes and stromal cells in the visceral fat [97]. In addition, PAI-1 promoter is responsive to metabolic and hormonal factors associated with obesity. For example, increase in PAI-1 levels is explained by pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6 [9]. These cytokines produced by adipocytes can regulate PAI-1 expression in the body. However, the impact of obesity on endothelial and platelet PAI-1 remains unknown.

In obese patients, insulin resistance is a common metabolic disturbance. Insulin resistance is associated with vascular inflammation and thrombosis before type 2 diabetes is diagnosed [9]. Interestingly, PAI-1-/- mice fed a high-fat or high-carbohydrate diet had favourable outcomes on insulin and glycemic measures [98], and protective effects against insulin resistance and obesity [99]. Thus, it remains unknown if PAI-1 dysregulation is causative or the result of metabolic derangements associated with obesity.

Often, patients with elevated PAI-1 levels and inflammatory markers progressed to diabetes [100]. It has been well established that patients with diabetes are at increased risk of coronary disease from a plethora

of purported mechanisms. In these patients, elevated levels of plasma PAI-1 has been consistently observed. Both proinsulin and products of insulin are elevated in type 2 diabetes and increases the expression of PAI-1 [101]. A large systematic review of 52 observational studies reported significantly elevated PAI-1 antigen levels in patients with type 2 diabetes compared to the controls [48]. In addition, 8 prospective studies included in the review showed a 67% increased risk of type 2 diabetes in the highest vs. lowest tertile of baseline PAI-1 levels [48]. Whether PAI-I is pathogenic in the observed increased risk of atherothrombotic events observed in patients with diabetes remains to be established.

1.6.3 PAI-1 and Atherosclerosis

Large studies have identified PAI-1 as a risk factor for the development and recurrence of atherosclerotic disease [37]. Previously, this relationship did not persist after adjusting for cardiac risk factors suggesting levels paralleled established risk factors. In a recent systematic review, a relationship between the highest PAI-1 antigen level on coronary artery disease risk (odds ratio (OR) = 1.22 per unit increase in log-transformed PAI-1; 95% CI: 1.01 – 1.47) independent of cardiac risk factors was identified [25]. In fact, the highest quantile of blood PAI-1 antigen level was associated with the highest risk of coronary disease compared to the lowest quantile (OR = 2.17; 95% CI: 1.13 – 1.88) [25]. Thus, current evidence supports a relationship between PAI-1 and disease presence without inferring clear causality.

Here, animal studies provide a possible causal relationship between PAI-1 and vascular events not afforded by previous epidemiologic association studies. Studies which evaluated the effect of PAI-1 knockout on atherosclerosis in mice revealed reduced atherosclerosis in the carotid artery bifurcation site in PAI-1 knockout mice (PAI-$1^{-/-}$/apo$E^{-/-}$) compared to their apo$E^{-/-}$ controls [102]. Similar results were seen when the same mice were fed a high-fat chow diet for 8 weeks to accelerate atherosclerosis development with a difference in atherosclerotic lesion surface area of 3.2 ± 1.0 mm^2 in the apo$E^{-/-}$ control vs. 0.42 ± 0.02 mm^2 in PAI-1 knockout mice [102]. In addition, greater amount of fibrin deposition was observed at the bifurcation sites in PAI-$1^{+/+}$ mice possibly due to turbulent flow [103] or due to the protective effects of PAI-

1 which functions to stabilize migratory cells [104]. In studies which examined atherosclerotic plaque tissue, PAI-1 mRNA had been found at elevated levels primarily around the base of the plaque [105]. In addition, mice with PAI-1 overexpression exhibited age-dependent spontaneous macrovascular coronary thrombosis and sub-endothelial myocardial infarction in the absence of other established risk factors [106]. Thus, PAI-1 appears to be influential in the development of both atherosclerosis and atherothrombosis and remains a promising target for novel therapies.

1.7 PAI-1 and the Post-PCI Artery

1.7.1 Epidemiology

Although the association between PAI-1 and coronary artery disease has been established, there remains a large gap of knowledge in PAI-1 being used as a biomarker after PCI. With the limited data that currently exists, it appears that elevated PAI-1 is associated with greater rates of all-cause mortality (hazard ratio (95% CI): 2.98 (1.10-8.06)) [107]. Thus, adequately powered studies to evaluate clinical outcomes such as target lesion failure are needed to ascertain if PAI-1 is useful to stratify patients undergoing PCI into high and low risk categories.

1.7.2 In-Stent Restenosis

In-stent restenosis (ISR) is a result of neointima formation or intimal thickening resulting in narrowing of the stented segment following PCI[108]. Despite considerable research in the field, our understanding of the molecular mechanisms behind the pathophysiology of ISR remain debated and have been reviewed in detail elsewhere[109]. Briefly, histological studies have shown an initial recruitment of inflammatory cells followed by smooth muscle cells and myofibroblasts which deposits an extracellular matrix leading to obstruction of the vascular lumen[109]. To reduce neointimal formation following PCI, drug-eluting stents were developed to prevent cellular proliferation and vascular inflammation. These drug eluting stents care made of a metallic backbone coated with polymers that elute immunosuppressive and

antiproliferative agents. However, drug eluting stents are not immune from ISR. In fact, Cassese *et al.* reported in patients with angiographic surveillance, restenosis occurred in 12.2% even in second-generation drug eluting stents [110]. Moreover, patients with in-stent restenosis presents as an acute coronary syndrome in 70% of cases –resulting in poor outcomes and dramatically increasing the rate of mortality [111]. Thus, identifying additional pathways for therapeutic intervention will improve patient outcomes.

Accordingly, conflicting data suggests PAI-1 plays a role in vascular remodelling and neointimal formation [61, 74]. Katsaros *et al*. [112] examined the relationship between PAI-1 activity levels (IU/mL) and in-stent restenosis in patients undergoing PCI with drug-eluting stents. Those in the lowest PAI-1 tertile were noted to have a 9.5-fold increased risk in ISR, independent of clinical risk factors [112]. In addition, the change in PAI-1 activity levels 24 hours post-PCI was much higher in patients who experienced in-stent restenosis, suggesting that acute change is associated with adverse events. On the surface, the role of PAI-1 influencing ISR appears to conflict the finding that elevated levels of PAI-1 is associated with target lesion failure. Target lesion failure is a composite of cardiac or unexplained death, target vessel myocardial infarction, and target vessel revascularization[113-118]. However, since cell migration can be reduced by PAI-1 ·vitronectin complex, it is mechanistically plausible that PAI-1 deficiency accelerates restenosis[119]. In addition, whole blood plasma PAI-1 levels may not accurately depict the locally elevated PAI-1 levels at the vascular site of injury. Indeed, in studies examining ISR in post-PCI with bare-metal stents, low levels or absence of PAI-1 was predictive of in-stent restenosis[120, 121]. With a lack of direct pathophysiological evidence at the site of stent deployment, studies are required to predict the relationship between PAI-1 levels and in-stent restenosis.

1.7.3 Stent Thrombosis

Stent thrombosis (ST) occurs when a thrombus forms within the stent lumen causing lumenal occlusion and myocardial infarction. ST occurs predominantly due to three reasons: (i) Incomplete stent apposition to the coronary lesion; (ii) Incomplete re-endothelialization and delayed healing of the stented vessel; (iii) inadequate antiplatelet administration[1]. The development of dual antiplatelet therapy in the form of acetylsalicylic acid and P2Y12 inhibitors to inhibit platelet activation and second-generation drug eluting stents has since reduced ST [122, 123]. Major risk factors to ST are platelet hyper-reactivity and diabetes. Risk factors – such as diabetes – place patients at high risk even if they respond to an antiplatelet regimen highlighting that additional factors remain increasing the atherothrombotic risk.

Since elevated PAI-1 levels have been associated with diabetes, an environment where platelet hyper-reactivity is prevalent, it stands to reason that augmented PAI-1 release from diabetic platelets increases the risk of stent thrombosis (Figure 1-3). In addition to elevated levels within the vascular milieu, anti-proliferative agents employed on drug eluting stents have been associated with induction of PAI-1 expression. In cultured human endothelial cells and mice, both paclitaxel and rapamycin enhanced PAI-1 transcript and protein expression – providing another means of augmented PAI-1 activity. [124] Indeed, elevated PAI-1 level presents an important mechanism towards the prothrombotic effects observed with drug eluting stents. Despite extensive studies conducted in endothelial cells, the effect of elevated PAI-1 levels has been poorly studied in platelet culture models. More studies are needed to elucidate the role of these anti-proliferative agents with platelet activation and PAI-1 release which may play a significant role in thrombosis.

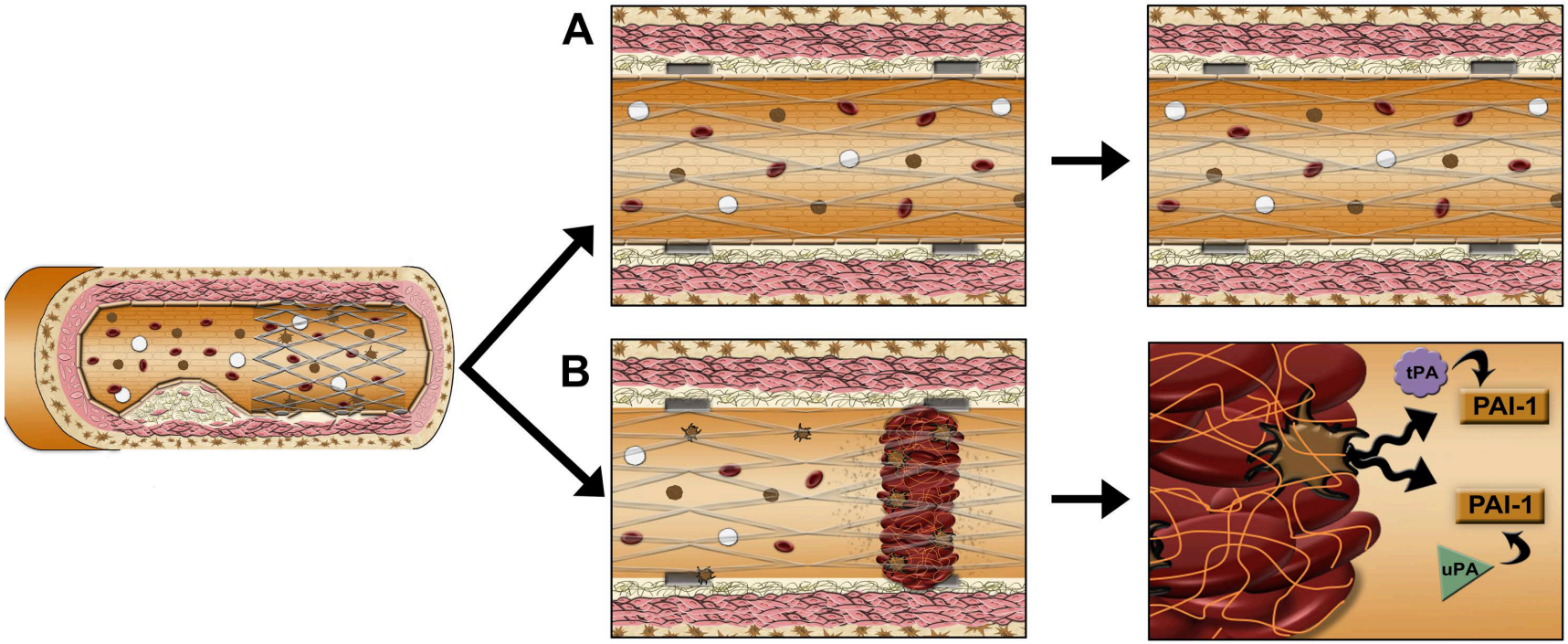


Figure 1-3. Stent thrombosis, a major complication of percutaneous coronary intervention.

(**A**) Normal re-endothelialization after stent placement signifying optimal coronary repair. (**B**) Stent thrombosis (ST) which occurs due to incomplete stent apposition or incomplete re-endothelialization of the stent. Increased rate of ST is observed in type 2 diabetes, which is associated with hyper-reactive platelets and elevated levels of plasma and platelet PAI-1 levels. Thus, an increase in local concentration of PAI-1 as a result of increased release of PAI-1 from platelets can favor the anti-fibrinolytic cascade to inhibit the endogenous fibrinolytic system (u-PA and t-PA) within the human body.

1.8 Therapeutic Targeting of PAI-1

Currently, coronary intervention involves a combination of mechanical reperfusion with medical therapies targeting both primary and secondary arms of the clotting cascade. Since platelet-rich arterial thrombi contain 2-3-fold greater platelet content than a venous equivalent, platelet PAI-1 release may enhance the anti-fibrinolytic capacity of the thrombus within coronary arteries. Thus, a combination of platelet and PAI-1 inhibition may prove beneficial to improving pharmacologic and mechanical thrombolysis and reperfusion.

To this end, examples in small preclinical studies have demonstrated therapeutic efficacy to inhibit PAI-1. Monoclonal antibodies designed against PAI-1 have shown thrombolytic efficacy in rat thrombosis models [125]. The drug acts by binding to allosteric sites preventing the interaction between PAI-1 and proteases [126]. A group screened and identified the natural aliphatic polyketide belonging to the Annonaceous acetogenins group, annonacinone, which was then experimentally verified to reveal a greater potency in an *in vitro* and *ex vivo* setting in human plasma [127]. Pre-incubation of human PAI-1 with annonacinone inhibits PAI-1 activity in tPA administration in thromboelastography. Several small molecule inhibitors of PAI-1 have been patented, but are yet to be advanced to clinical trials in humans [128]. Although the potency of PAI-1 inhibition has been well demonstrated in animal models, the multiple classes of PAI-1 inhibitors have yet been studied beyond the laboratory setting and have been discussed in detail in other reviews [128, 129]. However, due to the numerous and promising results shown in pre-clinical studies of the PAI-1 inhibitor tiplaxtinin, it will be the subject of discussion for the remainder of the review target lesion failure.

1.8.1 Tiplaxtinin

Tiplaxtinin (PAI-039), a small molecule PAI-1 antagonist, has shown efficacy in rodent [126] and canine [130] models of thrombolytic efficacy. Mutagenesis studies identified the binding site of tiplaxtinin to PAI-1 neighbouring the vitronectin binding site [131], explaining the ineffectiveness of the agent in inhibiting vitronectin-bound PAI-1 [127]. When vascular injury was induced, PAI-1 activity elevated 5-fold but was completely neutralized after the administration of tiplaxtinin. In contrast, without vascular injury,

administration of tiplaxtinin did not affect basal PAI-1 activity [132] which is critical as it could potentially affect bleeding events. Although tiplaxtinin is a promising agent, it has only been studied in animal models for thrombosis and lacks data as a complimentary therapy following stenting alone or in combination with antiplatelet regimens.

Tiplaxtinin was also studied for its effect of vascular smooth muscle and endothelial cell migration. Although it appears to have no effect on endothelial cell migration, tiplaxtinin inhibits smooth muscle cell migration and intimal hyperplasia in cell culture models and mechanical carotid artery injuries in mice [74]. Importantly, while it affects the negative aspects of vascular remodeling in the murine model, it did not affect re-endothelialization after mechanical vascular injury induction [74]. This promising result of tiplaxtinin allowing re-endothelialization while inhibiting smooth muscle cell migration seems optimal to reduce ISR and ST after stent deployment but has not been studied in large animal models with stent implantation.

1.9 Conclusion

PAI-1 continues to be studied for its role in the pathogenesis of vascular disease. Further advancements identify novel targets in the pathogenesis of disease and facilitate the development of novel therapeutic approaches.

1.10 Sources of Funding

R.G.J. was supported by the Vanier CIHR Canada Graduate Scholarship for his graduate studies.

1.11 Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest for the completion of the manuscript.

Chapter 2

Systematic Review of Plasminogen Activator Inhibitor-1 and Major Adverse Cardiac Events

2.1 Preface

This chapter has been previously published as a systematic review and meta-analysis article in *Thrombosis Journal*:

> **Jung RG**, Motazedian P, Ramirez FD, Simard T, Di Santo P, Visintini S, Faraz MA, Labinaz A, Jung Y, Hibbert B. Association between plasminogen activator inhibitor-1 and cardiovascular events: A systematic review and meta-analysis. *Thrombosis Journal*. 2018;16:12

2.2 Abstract

Introduction: Small studies have implicated plasminogen activator inhibitor-1 (PAI-1) as a predictor of cardiovascular events; however, these findings have been inconsistent.

Objective: To examine the potential role of PAI-1 as a marker for major adverse cardiovascular events (MACE).

Methods: We systematically reviewed all indexed studies examining the association between PAI-1 and MACE (defined as death, myocardial infarction, or cerebrovascular accident) or restenosis. EMBASE, Web of Science, Medline, and the Cochrane Library were searched through October 2016 to identify relevant studies, supplemented by letters to authors and review of citations. Studies reporting the results of PAI-1 concentration and/or activity levels in association with MACE in human subjects were included.

Results: Of 5961 articles screened, we identified 38 articles published between 1991 to 2016 that reported PAI-1 levels in 11,010 patients. In studies that examined PAI-1 antigen and activity levels, 15.1% and

28.2% of patients experienced MACE, respectively. Patients with MACE had higher PAI-1 antigen levels with a mean difference of 6.11 ng/mL (95% CI, 3.27-8.96). This finding was similar among patients with and without known coronary disease. Comparatively, studies that stratified by PAI-1 activity levels were not associated with MACE. In contrast, studies of coronary restenosis suggest PAI-1 antigen and activity levels are negatively associated with MACE.

Conclusion: Elevated plasma PAI-1 antigen levels are associated with MACE. Definitive studies are needed to ascertain if PAI-1 acts simply as a marker of risk or if it is indeed a bona fide therapeutic target.

2.3 Introduction

Obstructive coronary artery disease (CAD) is the leading cause of mortality in the western world. The cornerstone of therapy for CAD remains revascularization and secondary medical therapy to modify risk factors. The fibrinolytic system has implications for both approaches to disease management. First, percutaneous coronary intervention (PCI) with implantation of a coronary stent remains the predominant method of coronary revascularization [1]. However, complications such as in-stent restenosis and stent thrombosis following PCI limit its efficacy. Thus, in the peri-revascularization period, preventing thrombotic events is paramount until the vessel's endothelial lining and function are restored. Second, long term therapy with antiplatelet and/or oral anticoagulation is an integral part of secondary preventive medical therapy. Thus, dysregulation of the fibrinolytic pathways may increase the risk of complications from revascularization therapy *and* diminish the efficacy of long-term medical therapy to reduce the risk of recurrent events.

The fibrinolytic system is activated by the conversion of plasminogen to plasmin by serine proteases such as tissue or urokinase-type plasminogen activator (t-PA and u-PA, respectively). In contrast, fibrinolysis is inhibited by plasminogen activator inhibitor-1 (PAI-1), which is a member of the serine protease inhibitor (SERPIN) family. Ultimately, thrombosis risk is influenced by the balance between PAI-

1 and t-PA. Thus, an increase in the PAI-1 levels in plasma can induce a hypercoagulable state [133]. PAI-1 is released by vascular endothelial cells, hepatocytes, adipocytes, cardiomyocytes, fibroblasts, and platelets [48, 49]. In healthy humans, plasma levels of PAI-1 exceed t-PA by a ratio of over 4:1 with most of PAI-1 being cleared by the liver [9]. In pathologic conditions, PAI-1 production can be upregulated by pro-inflammatory factors such as TNFα, TGFβ, and insulin [134]. Elevated plasma PAI-1 levels have been associated with impaired fibrinolytic activity in stroke and coronary artery disease [135]. Moreover, PAI-1 antigen and activity levels are elevated in patients with type 2 diabetes [81], hyperinsulinemia [136], and those with insulin resistance [83, 137]. Yet, a definitive evaluation of the impact of elevated PAI-1 as a biomarker or therapeutic target has yet to be evaluated. Accordingly, we performed a systematic review and meta-analysis of PAI-1 antigen and activity levels and their relationship with major adverse cardiovascular events (MACE) in humans.

2.4 Methods

2.4.1 Literature Search Strategy

Literature searches were guided by a medical librarian with expertise in systematic reviews (S.V.) using a combination of key terms and index headings related to PAI-1, coronary disease (informed by the Cochrane review search strategy for coronary heart disease in exercise-based cardiac rehabilitation [138]), and the Cochrane Highly Sensitive search strategy to eliminate articles on animal studies in Medline. The search was additionally peer-reviewed by a second medical librarian (R.S.). Once finalized, the search strategy was then translated to other bibliographic databases. The final search was conducted on October 2016 in Medline (Ovid) (In-Process & Other Non-Indexed Citations and Ovid MEDLINE(R) 1946-), Embase (Ovid) (Embase Classic + Embase 1947-), Cochrane Library (Ovid) (from inception), and Web of Science (Thomson Reuters) (all indexes, from inception). Search results were exported to EndNote X7 (Thomson Reuters, New York, USA) and duplicates eliminated using the program's duplicate identification feature and manual inspection. A review protocol was produced but not registered in a database.

Titles and abstracts were screened by two independent reviewers (R.J. and P.M.) using Covidence (Melbourne, Australia). Full articles were retrieved in cases of missing abstracts. Corresponding authors were contacted for additional information when necessary.

2.4.2 Inclusion and Exclusion Criteria and Quality Assessment

Studies were included if they met the following criteria: (1) PAI-1 antigen or activity levels were reported; (2) the population studied comprised individuals aged 18 years or older; (3) components of MACE (death, myocardial infarction, and cerebrovascular events including stroke and transient ischemic attacks) or restenosis were reported; (4) articles were published in English. Exclusion criteria included: (1) PAI-1 polymorphism studies examining the association between 4G/5G and adverse events; (2) animal or *in vitro* studies; and (3) studies reporting hazard ratios only. Full text data extraction was conducted by two independent evaluators (R.J. and P.M.). Each reviewer independently extracted patient population characteristics, group sizes, PAI-1 antigen and activity levels, follow-up duration, and MACE and restenosis. All discrepancies were resolved by consensus prior to locking the database for analysis (Table 2-1 and Table 2-2). Included observational studies were evaluated for quality and risk of bias using the Newcastle-Ottawa Quality Assessment Scale [139] by two independent evaluators (R.J. and P.M.) with disagreements resolved by consensus. Visual funnel plot inspection was used to screen for publication bias.

2.4.3 Statistical Analysis

The primary clinical endpoint for this study was MACE – a composite of death, myocardial infarction, or cerebrovascular events. The secondary endpoint included components of the primary as well as coronary restenosis in patients undergoing coronary revascularization. Mean circulating PAI-1 antigen (ng/mL) and activity (IU/mL) levels and their associated standard deviations were used for analyses. Fourteen studies reported median and interquartile ranges, which were converted to approximated means and standard deviations using the method described by Wan et al. [140].

All analyses were performed using Review Manager (RevMan) 5.3 (Cochrane Collection, Copenhagen, Denmark). PAI-1 antigen and activity levels were compared between patients with or without the outcomes of interest either as absolute values or dichotomized as high vs. low. Random effects models stratified by study design and study quality were used to generate pooled mean differences with 95% confidence intervals. *Post-hoc* meta regression was performed to account for timing of blood draw and acute phase reactions in studies of patients presenting with acute myocardial infarction or stroke.

Table 2-1. Studies reporting PAI-1 antigen levels (ng/mL) and major adverse cardiovascular events and restenosis

					Event							No Event		
Reference	Year	Study Design	Follow-up (months)	Population of interest	N	PAI-1 (IU/mL)	Range	Death	MI	Restenosis	CVA	N	PAI-1 (IU/mL)	Range
Sane et al. [50]	1991	Cohort	24	Fibrinolytics	24	57	58	24	n/a	n/a	n/a	315	54	53
Cortellaro et al. [51]	1993	Case-Control	24	Subgroups Combined	58	11.3	0.7	13	17	n/a	20	87	7.3	0 5
Brannstrom et al. [52]	1995	Cohort	46	Anticoagulant	38	21.9	15.1	38	n/a	n/a	n/a	167	16.7	11.8
Juhan-Vague et al. [53]	1996	Cohort	24	MI	106	18.2	8 2	40	66	n/a	n/a	2700	14.8	8.9
Nordt et al. [54]	1998	Cohort	12	Fibrinolytics	5	28.8	14.3	n/a	n/a	5	n/a	26	27.1	15.4
Alaigh et al. [55]	1998	Cohort	6	Elective PCI	28	20.75	11.06	n/a	n/a	28	n/a	45	24.5	13.85
Moss et al. [56]	1999	Cohort	26	MI	81	25	18	25	56	n/a	n/a	964	29	28
Redondo et al. [57]	2001	Cohort	24	MI	37	40.725	22.28	2	5	30	n/a	157	42.65	21.02
Fornitz et al. [58]	2001	Cohort	6	Elective PCI	7	82.6	26.6	n/a	n/a	7	n/a	12	72.2	27
Bogaty et al. [59]	2001	Case-Control	48	MI	23	23.83	19.04	n/a	8	n/a	n/a	77	18.9	16.24
Ganti et al. [60]	2002	Cohort	n/a	MI	4	80.68	16.38	4	n/a	n/a	n/a	38	61	21.95
Lip et al. [61]	2002	Cohort	12	Stroke	27	56.5	30.7	27	n/a	n/a	n/a	59	45.9	23.3
Inoue et al. [62]	2003	Cohort	6	MI	24	28	4	n/a	n/a	24	n/a	42	29	4
Christ et al. [20]	2005	Cohort	6	Elective PCI	25	14.8	0.7	n/a	n/a	25	n/a	55	16.8	2.1
Robinson et al. [63]	2007	Cohort	5 to 51	Coronary Heart Disease	19	36.3	17.9	2	2	n/a	2	79	45.4	25.6
Katsaros et al. [24]	2008	Cohort	6 to 8	Elective PCI	12	11.69	8.05	n/a	n/a	12	n/a	61	22.78	18.76
Thogersen et al. [64]	2009	Case-Control	n/a	Healthy	50	38.27	16.79	n/a	50	n/a	n/a	56	29	15.75
Akkus et al. [65]	2009	Cohort	12	Cardiogenic Shock	33	116.5	97.26	33	n/a	n/a	n/a	27	71.33	54.79
Pineda et al. [66]	2010	Cohort	36	MI	25	65.13	53.3	4	21	n/a	n/a	117	70.1	48.34
Wennberg et al. [67]	2012	Case-Control	168	Healthy	469	57.28	25.83	n/a	469	n/a	n/a	895	51.23	25.11
Yano et al. [68]	2013	Cohort	20	Smokers	66	65.73	64.57	n/a	11	n/a	55	744	42.97	36.54
Yano et al. [69]	2014	Cohort	30	Hypertension	42	31.67	16.12	4	13	n/a	16	548	28.33	14.87
Knudsen et al. [70]	2014	Case-Control	12	HIV	54	111	8.5	3	51	n/a	n/a	54	92	7
Golukhova et al. [71]	2015	Cohort	28	Elective PCI	23	72.75	29.86	2	9	11	n/a	71	49.75	23.16

CVA cerebrovascular accident

Table 2-2. Studies reporting PAI-1 activity levels (U/mL) and major adverse cardiovascular events and restenosis

					Event						No Event		
Reference	Year	Study Design	Follow-up (months)	Population of interest	N	PAI-1 Activity (U/mL)	Range	Death	MI	Restenosis	N	PAI-1 Activity (U/mL)	Range
Sane et al. [50]	1991	Cohort	24	Fibrinolytics	28	17	16	n/a	n/a	28	328	19	21
Shah et al. [72]	1992	Cohort	9	Elective PCI	28	8	7.1	n/a	n/a	28	40	12	8
Gray et al. [73]	1993	Cohort	0.1	MI	13	20.6	11	n/a	13	n/a	85	20.1	7.9
Malmberg et al. [74]	1994	Case-Control	90	MI	53	23.75	13.34	20	33	n/a	55	18	10.97
Brack et al. [75]	1994	Cohort	4	Elective PCI	16	4.63	4.71	n/a	n/a	16	30	5.77	5.06
Nordt et al. [54]	1998	Cohort	12	Fibrinolytics	5	8.7	8.3	n/a	5	n/a	26	9.9	8.2
Jansson et al. [76]	1998	Cohort	120	MI	54	9.1	5.1	54	n/a	n/a	69	10.6	7.1
Wiman et al. [77]	2000	Case-Control	3	MI	61	22.1	17.5	n/a	61	n/a	95	18.2	16.5
Wiman et al. [77]	2000	Case-Control	3	MI	25	15.4	13.6	n/a	25	n/a	38	17.8	12.4
Prisco et al. [78]	2001	Cohort	18	MI	18	11.27	7.64	n/a	n/a	18	36	15.8	27.49
Prisco et al. [78]	2001	Cohort	18	Elective PCI	6	8.33	8.1	n/a	n/a	6	42	7.57	9.52
Sargento et al. [79]	2003	Cohort	12	MI	7	6.34	1.56	5	2	n/a	80	4.47	1.84
Marcucci et al. [19]	2006	Case-Control	22 2	MI	109	22	9.09	54	55	n/a	411	24.25	10.63
Schoebel et al. [80]	2008	Cohort	2	MI	18	3.7	1.8	n/a	n/a	18	42	5.3	3.2

Wiman et al. [77] is presented twice as data for men and women were reported separately

2.5 Results

2.5.1 Included Studies – Study Selection

After excluding duplicate articles, 5,961 titles and abstracts were screened, of which 340 underwent full review and 38 were ultimately included (Figure 2-1, Table 2-1 and Table 2-2). Study populations were heterogeneous, including patients presenting with stable angina, acute coronary syndrome, and non-cardiac diseases. Study sample sizes ranged from 19 to 2,806. Most studies were of moderate quality (see Table 2-3 and Table 2-4 for details of study quality assessments). Funnel plots are shown in Figure 2-2 and Figure 2-3. In pooled analyses of all studies reporting PAI-1 antigen levels (n = 8999), 1362 events were reported, including 234 deaths, 823 myocardial infarctions, 101 cerebrovascular events, and 142 restenoses (Table 2-1). In all studies that examined PAI-1 activity levels (n = 1462), 413 events were reported, including 133 deaths, 194 myocardial infarctions, and 114 cases of restenosis (Table 2-2).

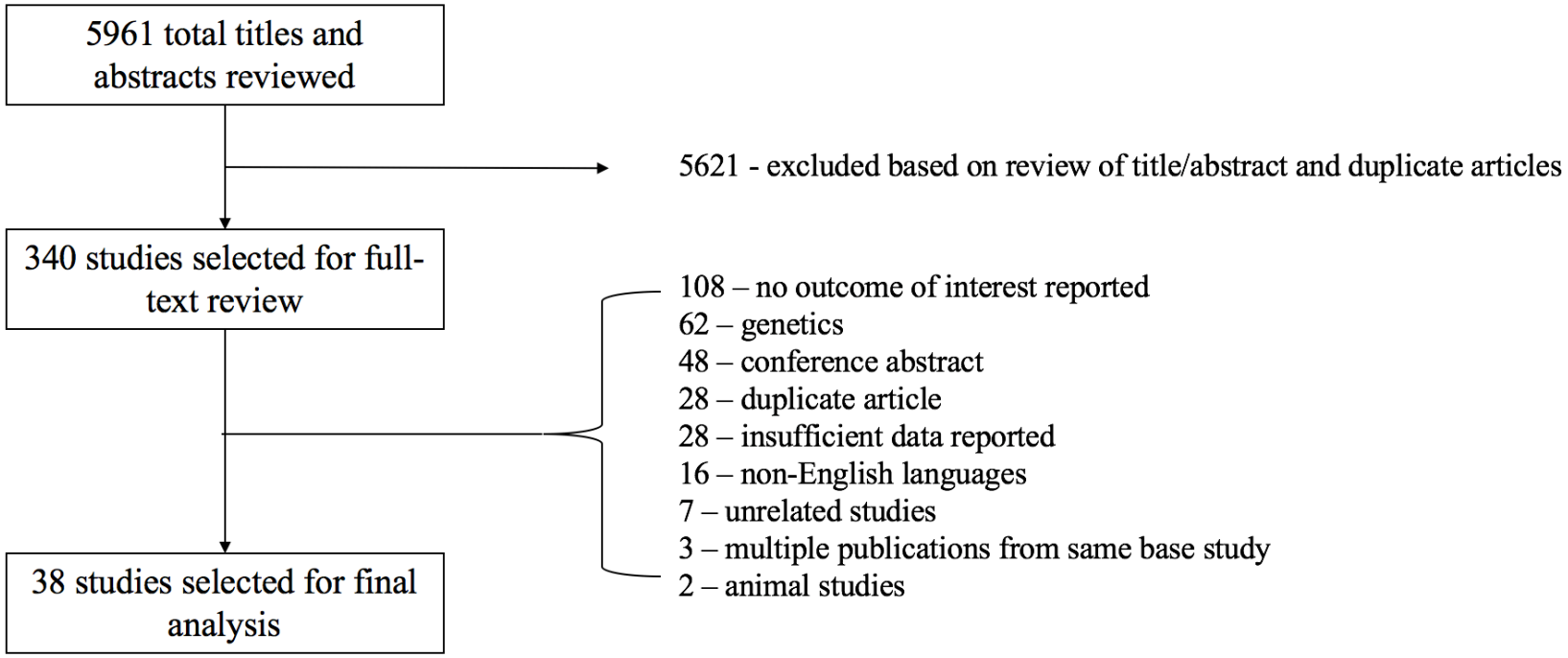


Figure 2-1. Flow diagram of the included PAI-1 studies for meta-analysis

Table 2-3. Newcastle-Ottawa Quality Assessment Scale for Observational Studies of PAI-1 concentration levels (ng/mL)

Reference	Year	Study Design	Selection	Comparability	Exposure Outcome	Total
Sane et al.[141]	1991	Cohort	3	0	2	5
Cortellaro et al.[142]	1993	Case-Control	2	2	3	7
Brannstrom et al.[143]	1995	Cohort	3	0	3	6
Juhan-Vague et al.[144]	1996	Cohort	3	0	3	6
Nordt et al.[145]	1998	Cohort	3	0	3	6
Alaigh et al.[146]	1998	Cohort	2	0	2	4
Moss et al.[147]	1999	Cohort	2	0	3	5
Redondo et al.[148]	2001	Cohort	3	0	2	5
Fornitz et al.[149]	2001	Cohort	2	0	2	4
Bogaty et al.[150]	2001	Case-Control	2	2	2	6
Ganti et al.[151]	2002	Cohort	2	0	1	3
Lip et al.[152]	2002	Cohort	3	0	3	6
Inoue et al.[153]	2003	Cohort	3	0	2	5
Christ et al.[154]	2005	Cohort	3	0	3	6
El-Menyar et al.[155]	2006	Cohort	3	0	2	5
Robinson et al.[156]	2007	Cohort	3	0	3	6
Katsaros et al.[112]	2008	Cohort	3	0	2	5
Thogersen et al.[157]	2009	Case-Control	4	2	2	8
Akkus et al.[158]	2009	Cohort	3	0	2	5
Arikan et al.[159]	2009	Cohort	3	0	3	6
Pineda et al.[160]	2010	Cohort	3	0	3	6
Wennberg et al.[161]	2012	Case-Control	4	2	3	9
Yano et al. (2013)[162]	2013	Cohort	3	0	3	6
Iacoviello et al.[163]	2013	Cohort	4	1	3	8
Yano et al. (2014)[164]	2014	Cohort	3	0	3	6
Knudsen et al.[165]	2014	Case-Control	2	2	1	5
Golukhova et al.[166]	2015	Cohort	2	0	3	5

Table 2-4. Newcastle-Ottawa Quality Assessment Scale for Observational Studies of PAI-1 activity levels (U/mL)

Reference	Year	Study Design	Selection	Comparability	Exposure/ Outcome	Total
Shah et al.[167]	1992	Cohort	3	0	2	5
Gray et al.[168]	1993	Cohort	2	0	2	4
Malmberg et al.[169]	1994	Case-Control	3	2	3	8
Brack et al.[170]	1994	Cohort	3	0	1	4
Jansson et al.[171]	1998	Cohort	1	0	3	4
Sinkovic et al.[172]	2000	Cohort	2	0	2	4
Wiman et al.[173]	2000	Case-Control	3	2	3	8
Prisco et al.[174]	2001	Cohort	2	0	3	5
Sargento et al.[175]	2003	Cohort	2	0	3	5
Marcucci et al.[176]	2006	Case-Control	2	2	3	7
Schoebel et al.[177]	2008	Cohort	2	0	3	5

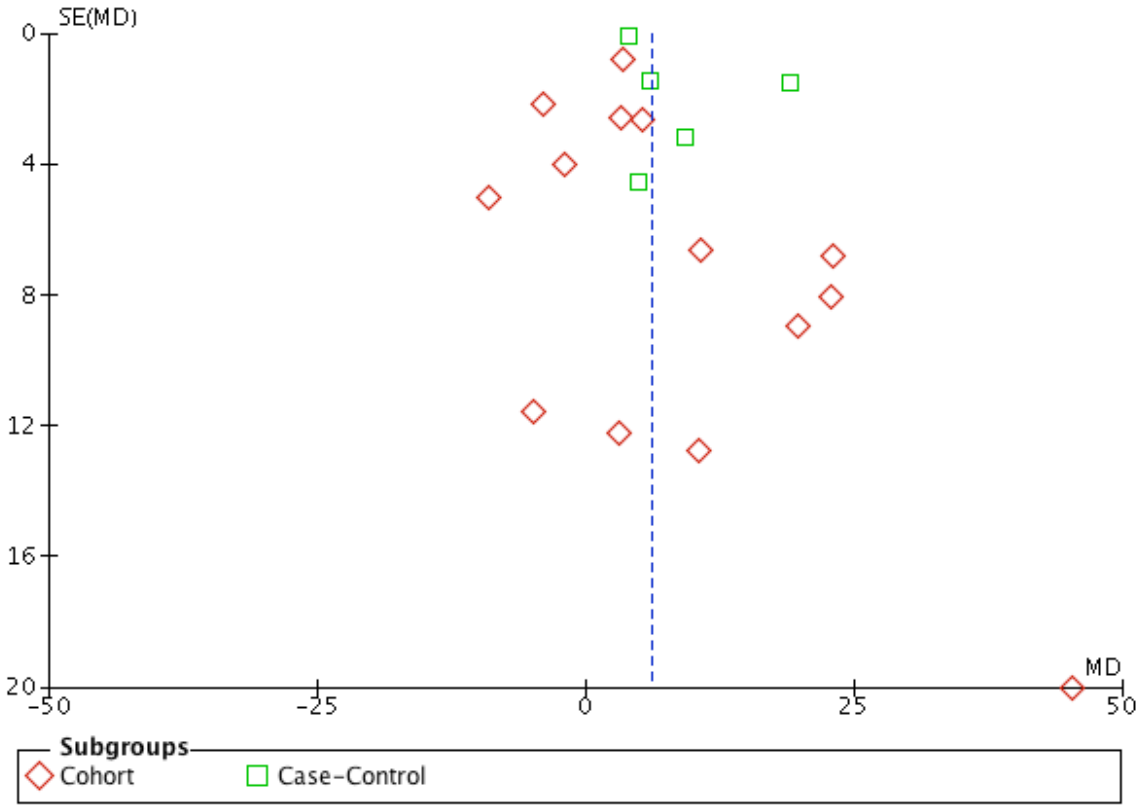


Figure 2-2. Funnel plots for studies examining PAI-1 antigen levels (ng/mL) and MACE

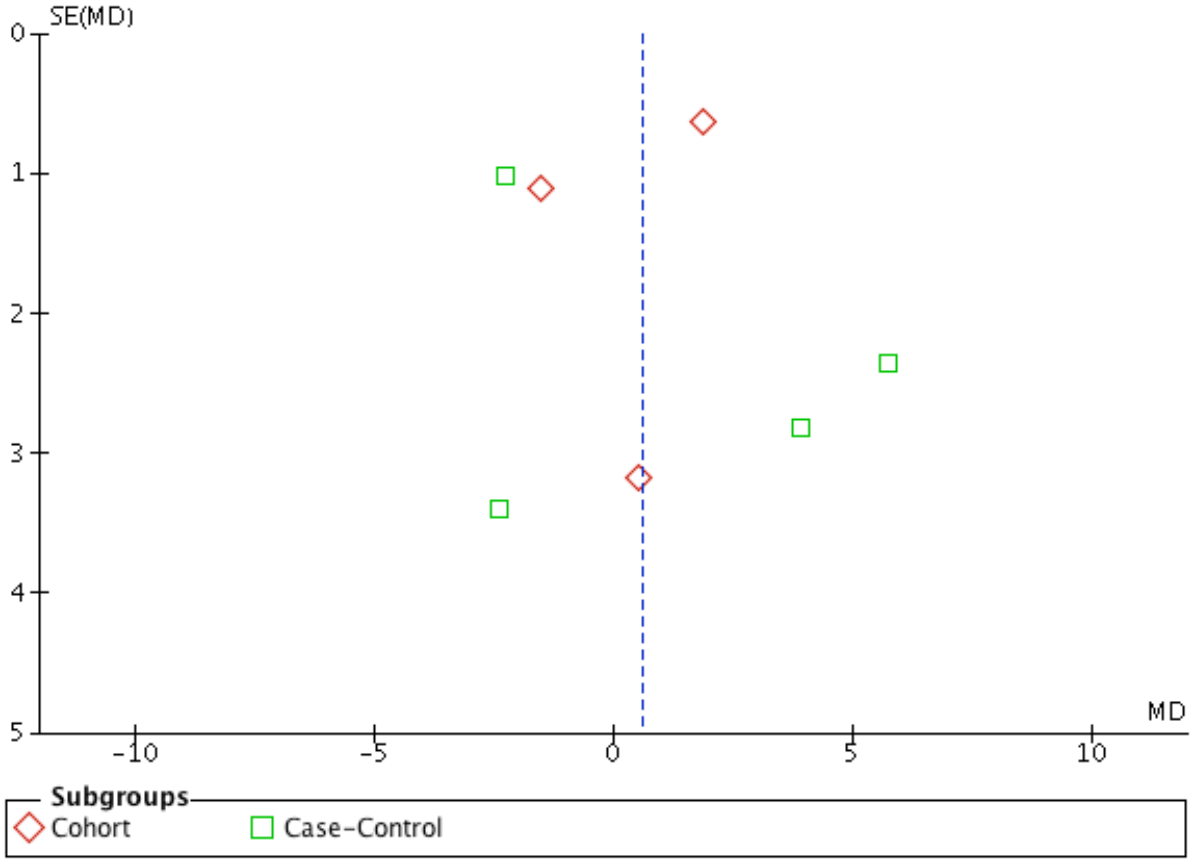


Figure 2-3. Funnel plots for studies examining PAI-1 activity levels (IU/mL) and MACE

2.5.2 PAI-1 and Major Adverse Cardiac Events

PAI-1 antigen levels were higher in those with MACE with a mean difference of 6.11 ng/mL (95% CI, 3.27-8.96, *P*<0.001) – a difference that was present irrespective of study design (Figure 2-4). When restricted to high-quality studies, PAI-1 antigen levels in patients with MACE were 5.22 ng/mL (95% CI, 2.97-7.54, P<0.001; Figure 2-5). Among seven studies reporting morning blood draws between 7:00 and 10:00 am, PAI-1 antigen levels were higher in those with MACE with a mean difference of 4.61 ng/mL (95% CI, 1.49-7.74, P=0.004; Figure 2-6). Meta-regression analysis of timing of the blood draw and acute phase studies was not predictive of the heterogeneity in our selected studies nor did it contribute to a greater understanding of the impact of PAI-1 in its association with MACE (Table 2-5).

In contrast to PAI-1 plasma antigen levels, there was no significant difference in PAI-1 activity levels between those with vs. without MACE (mean difference 0.59 IU/mL (95% CI, -1.63-2.80, *P*=0.60; Figure 2-7). No association between PAI-1 activity levels and MACE was observed when the analysis was restricted to three high-quality studies with a mean difference of 1.14 IU/mL (95% CI, -3.37-5.65, P=0.62; Figure 2-8).

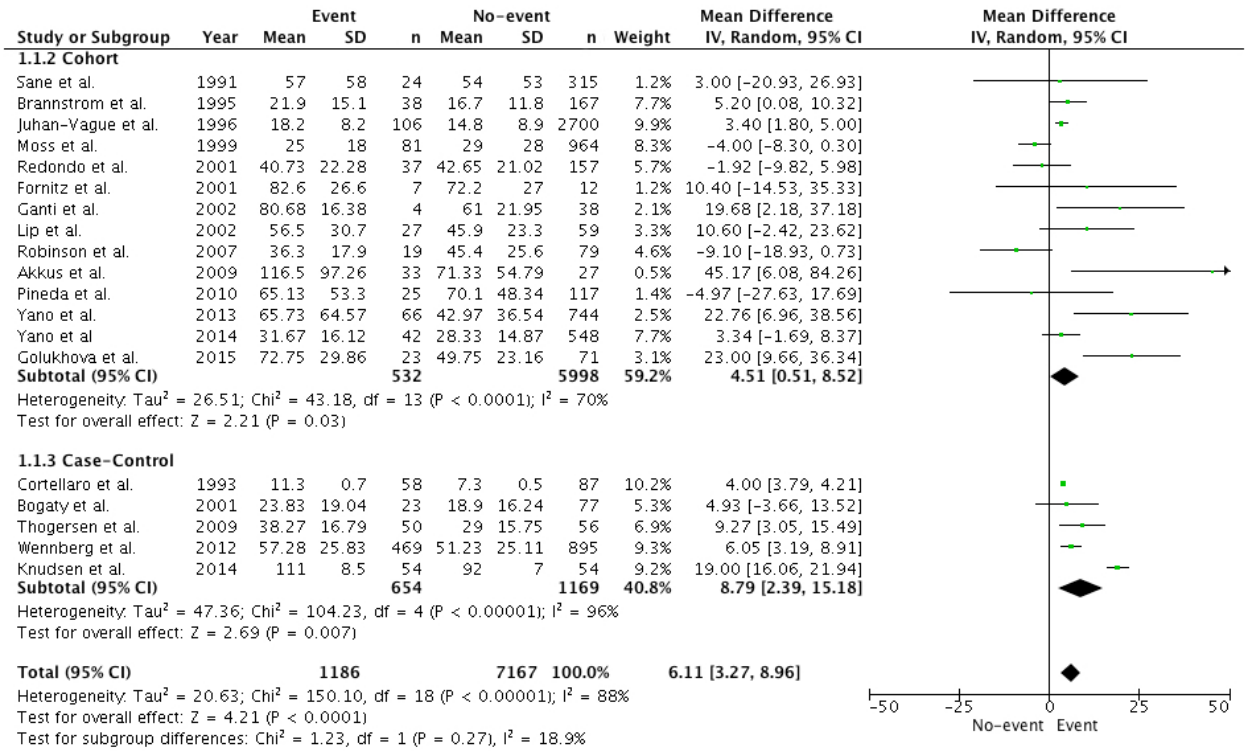


Figure 2-4. Comparison of mean PAI-1 antigen levels (ng/mL) in patients with major adverse cardiac events and control patients.

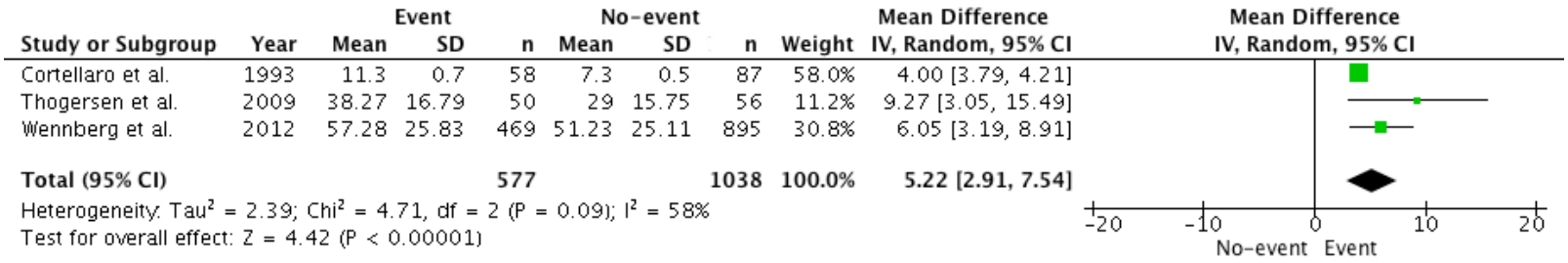


Figure 2-5. Comparison of mean PAI-1 antigen levels (ng/mL) and major adverse cardiac events in high-quality studies utilizing the Newcastle-Ottawa Scale

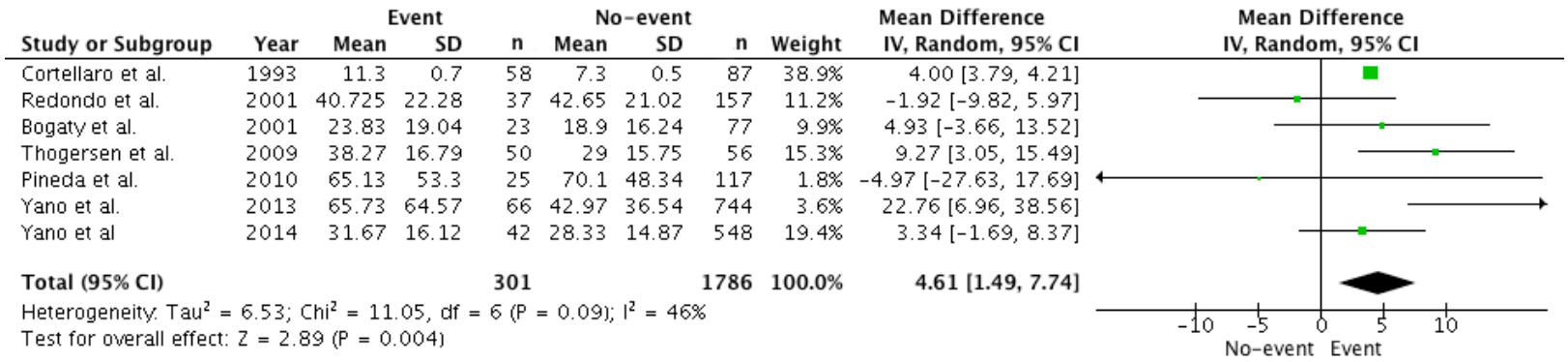


Figure 2-6. Comparison of mean PAI-1 antigen levels (ng/mL) in studies which reported morning blood draws ranging from 7:00-10:00 am and major adverse cardiac events

Table 2-5. Meta-regression results or the effect of time of blood draw and acute phase reaction on PAI-1 antigen and activity levels

	PAI-1 antigen levels (ng/mL)							PAI-1 activity levels (U/mL)						
Model	**N**	**Coeff.**	**95% CI**	**Tau2**	**Adj-R^2(%)**	**I^2 (%)**	**p**	**N**	**Coeff.**	**95% CI**	**Tau2**	**Adj-R^2(%)**	**I^2 (%)**	**p**
All covariates	24			65.98	-11.22	93.22	0.72	14			4.502	-35.39	55.86	0.9
Time of blood draw	24	-3.07	(-12.60, 6.47)				0.51	14	0.54	(-3.15, 4.22)				
Acute phase	24	1.66	(-9.35, 12.67)				0.757	14	0.54	(-3.06, 4.15)				

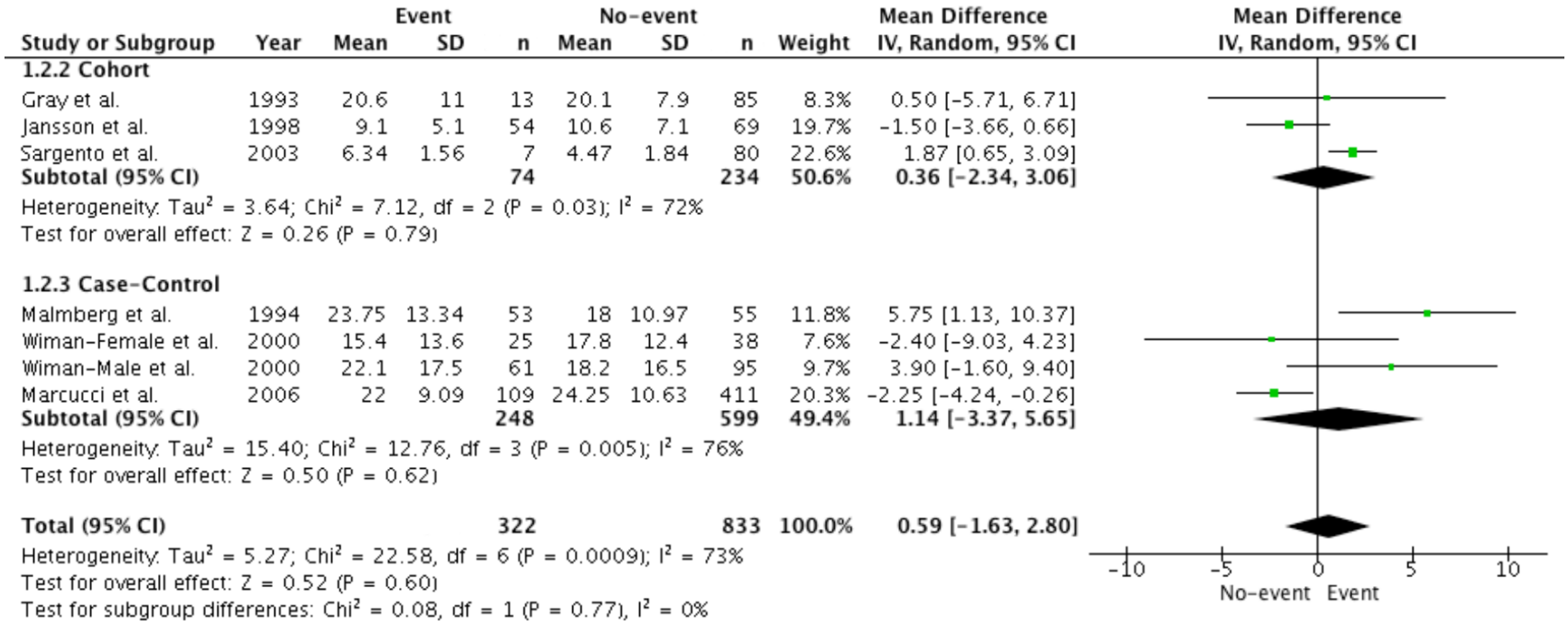


Figure 2-7. Comparison of mean PAI-1 activity levels (U/mL) in patients with major adverse cardiac events and control patients. Data is expressed as a mean difference and analyzed using a random effects model

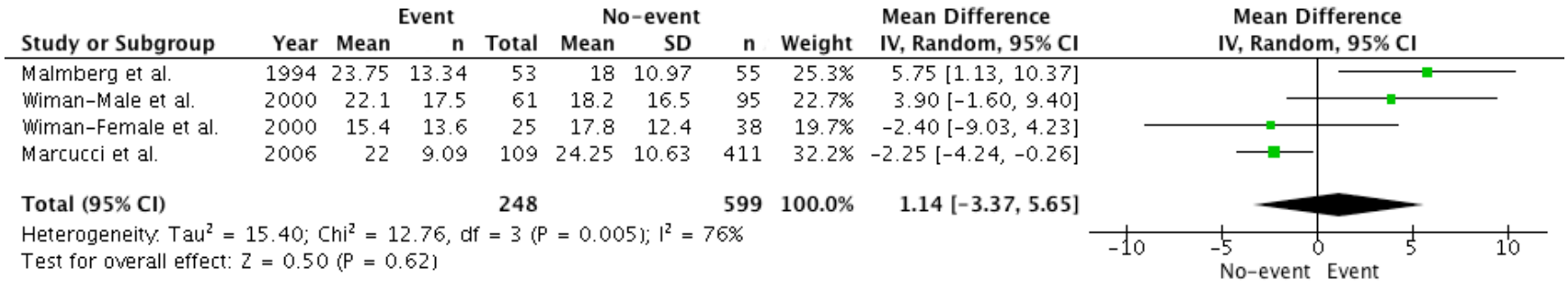


Figure 2-8. Comparison of mean PAI-1 activity levels (IU/mL) and major adverse cardiac events in high-quality studies utilizing the Newcastle-Ottawa Scale

2.5.3 Major Adverse Cardiovascular Events: Pre-Existing Versus no Known Coronary Artery Disease

Overall, among patients without prior coronary artery disease, 12.2% had an event. PAI-1 antigen levels in those without previously known coronary artery disease were on average 6.44 ng/mL higher in those with MACE relative to those without (95% CI, 2.64-10.25, $P<0.001$; Figure 2-9A). In studies that included patients with known CAD, 19.3% had MACE. PAI-1 antigen levels in those with known CAD were on average 5.49 ng/mL higher in those with MACE than those without (95% CI, 0.36-10.63, $P=0.04$; Figure 2-9B). No difference in PAI-1 activity levels between event and control groups was observed in the five studies reporting PAI-1 activity levels and MACE (Figure 2-10).

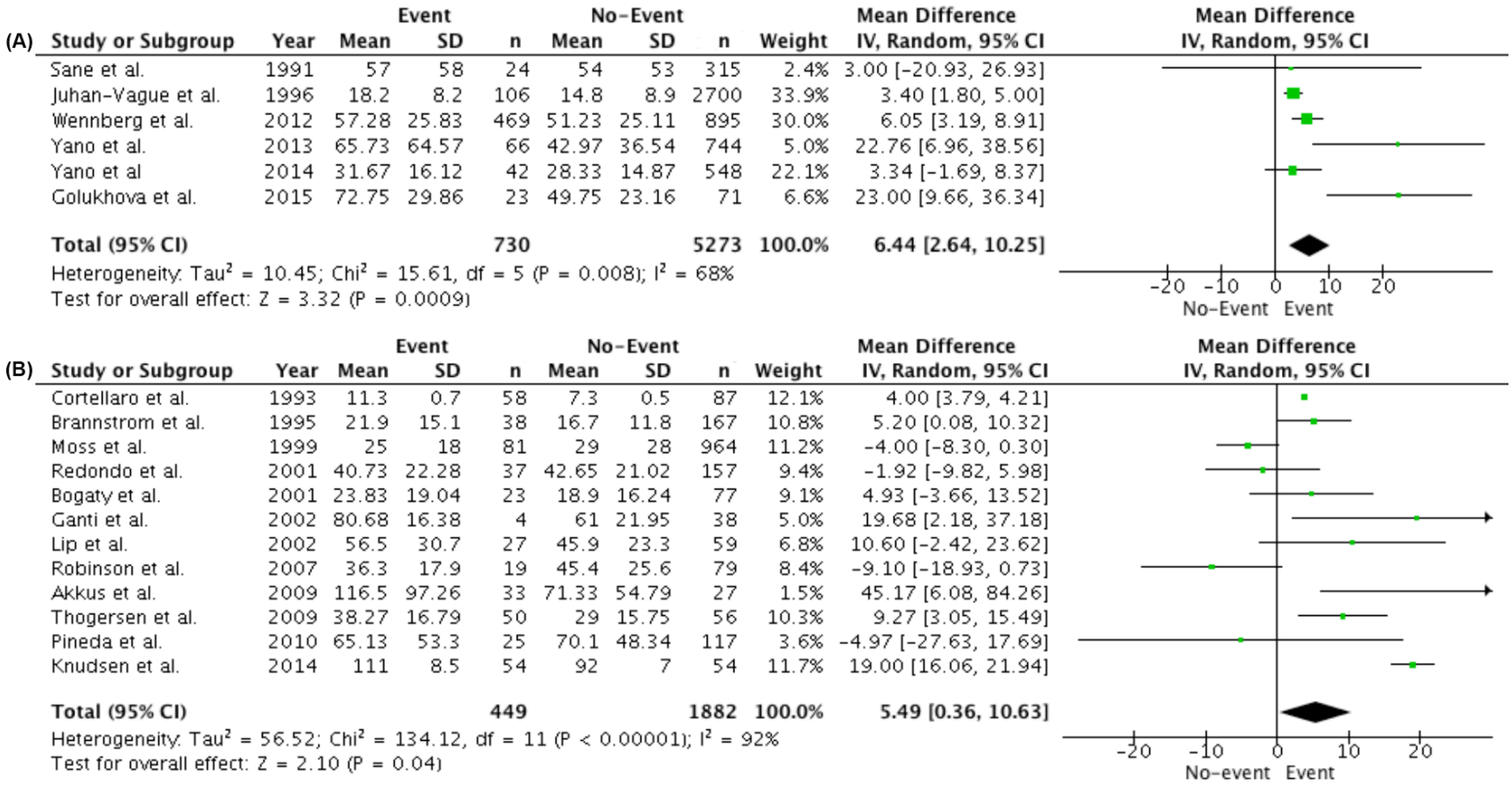


Figure 2-9. Comparison of mean PAI-1 antigen levels (ng/mL) in patients with primary and secondary major adverse cardiac events and control patients.

(**A**) PAI-1 levels (ng/mL) in patients with primary major adverse cardiac events. (**B**) PAI-1 levels (ng/mL) in patients with secondary major adverse cardiac events. Data is expressed as a mean difference and analyzed using a random effects model

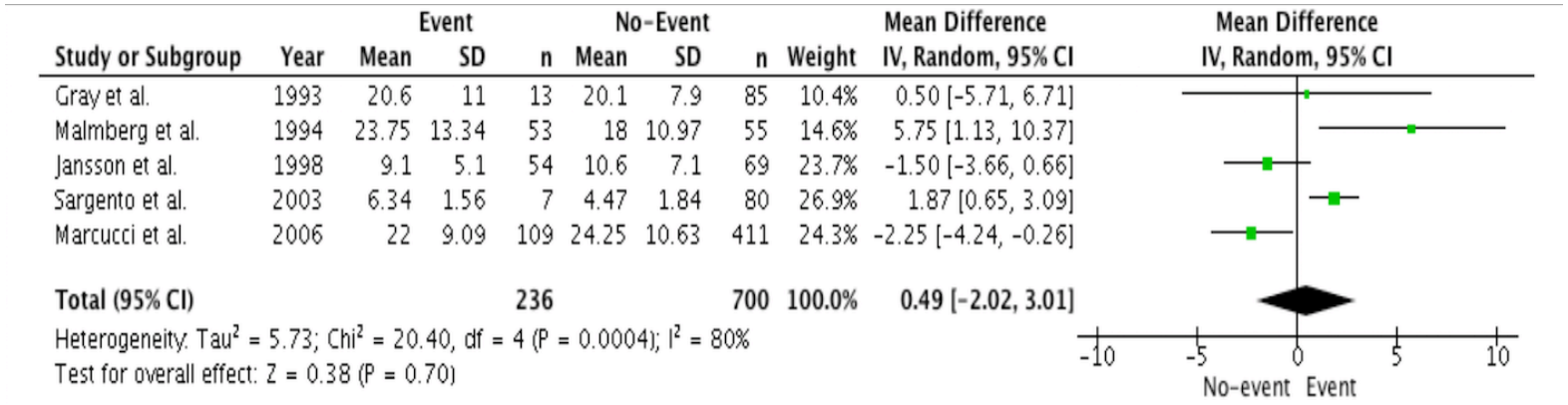


Figure 2-10. Comparison of mean PAI-1 activity levels (U/mL) in patients with secondary major adverse cardiac events and control patients

2.5.4 Death

In the five studies that reported mortality data, 126 deaths were observed (17.2% of patients). PAI-1 antigen levels were higher among patients who died (mean difference: 10.34 ng/mL (95% CI, 1.90-18.79, *P*=0.02; Figure 2-11).

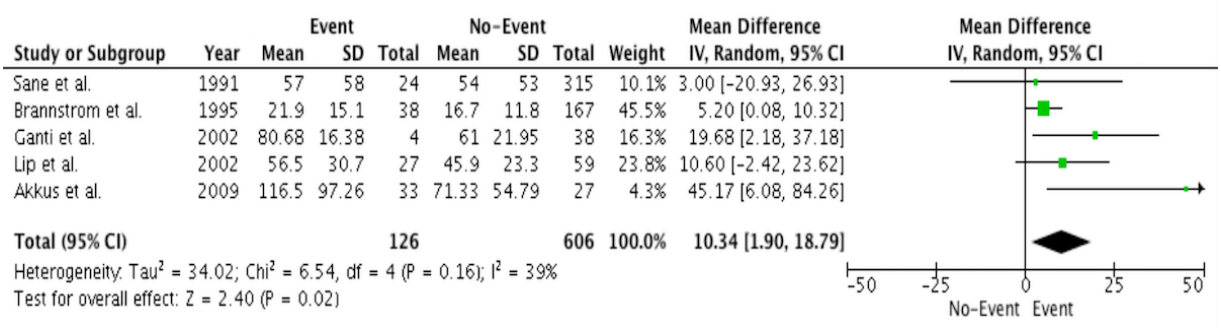


Figure 2-11. Comparison of mean PAI-1 antigen levels (ng/mL) in event and control patients for mortality

2.5.5 Restenosis

In the six studies that examined restenosis following percutaneous coronary intervention (with and without coronary stent implantation), 101 events were observed (29.5% of patients). PAI-1 antigen levels were lower in those with restenosis with a mean difference of -2.43 ng/mL (95% CI, -4.48-(-0.37), *P*=0.02; Figure 2-12A). An additional six studies provided restenosis rates and PAI-1 activity levels. These studies reported restenosis in 119 patients (17.9%). PAI-1 activity was lower in those with restenosis with a mean difference of -1.73 IU/mL (95% CI: -2.80-(-0.67), *P*=0.001; Figure 2-12B).

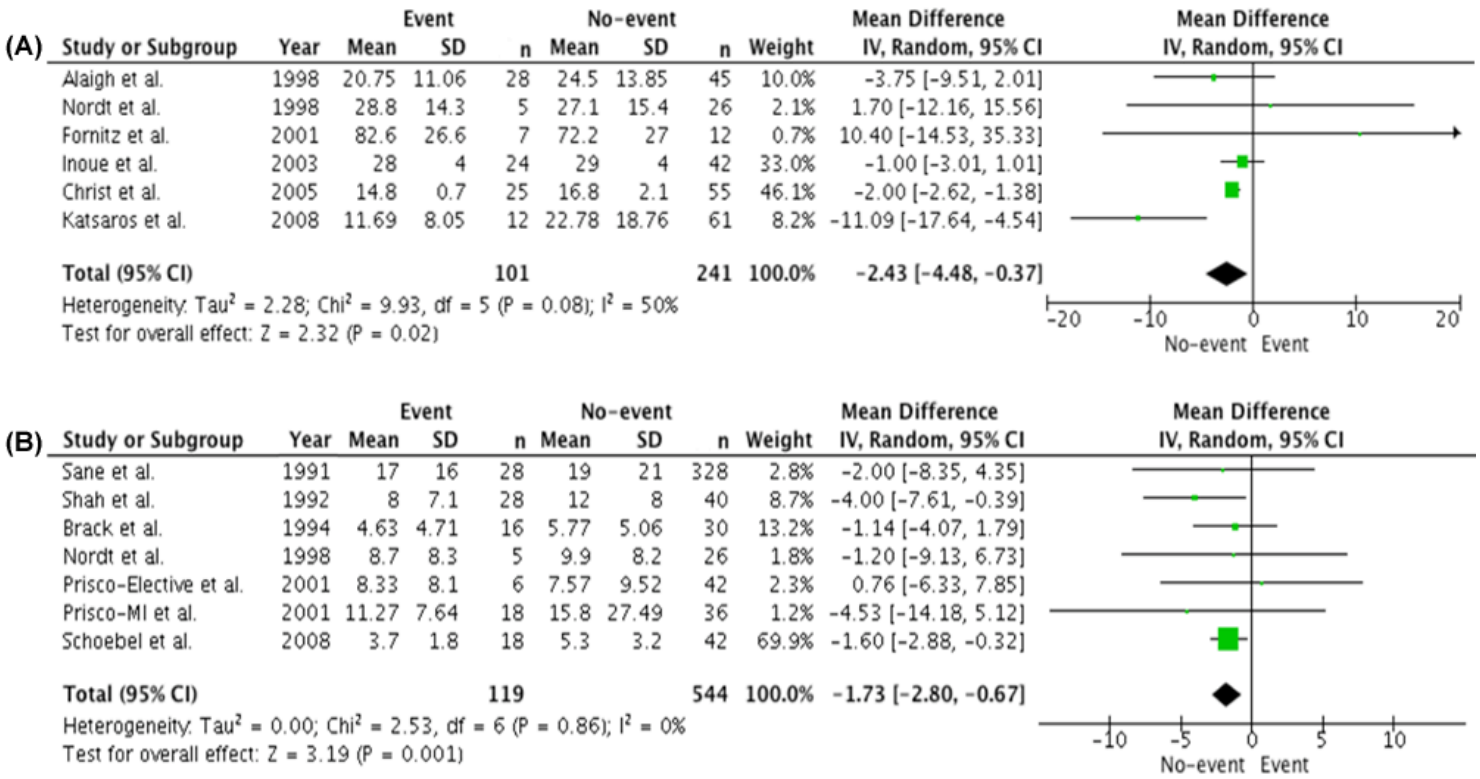


Figure 2-12. Comparison of mean PAI-1 antigen and activity levels in patients with restenosis and control patients. (**A**) Comparison of mean PAI-1 concentration levels (ng/mL) in patients with restenosis. (**B**) Comparison of mean PAI-1 activity levels (U/mL) in patients with restenosis. Data is expressed as a mean difference and analyzed using a random effects model

2.5.6 High Versus Low PAI-1 Levels

Three studies stratified their data by high versus low PAI-1 antigen levels. These studies reported a MACE rate of 54.4%. High PAI-1 antigen levels were associated with a 58% greater risk of MACE compared to low PAI-1 antigen levels (RR 1.58, 95% CI: 1.42-1.76, $P<0.0001$; Figure 2-13).

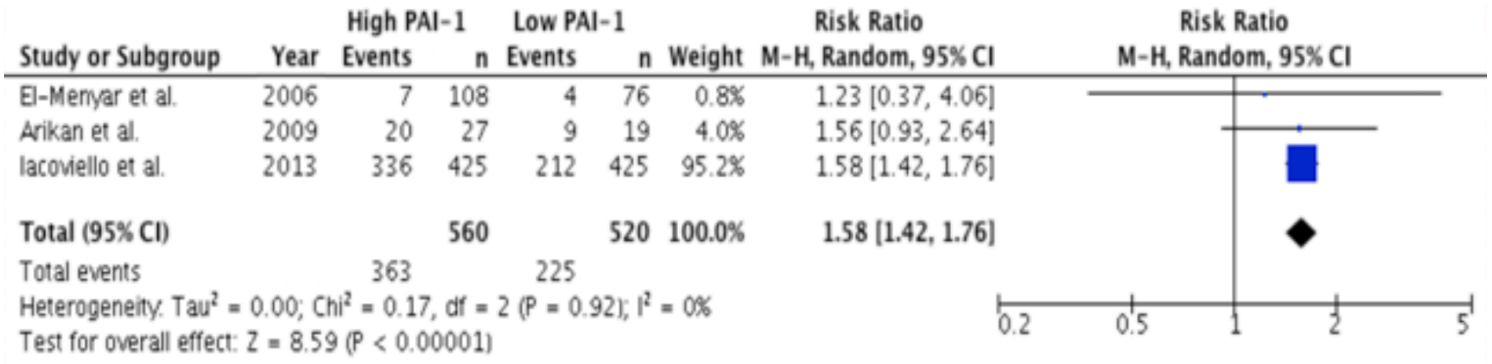


Figure 2-13. Comparison of risk of major adverse cardiac events in patients stratified by PAI-1 antigen levels (ng/mL). Data is expressed as a risk ratio and analyzed using a random effects model

2.6 Discussion

Incident and recurrent cardiovascular events remain important adverse outcomes despite major advances in revascularization and medical therapy. PAI-1 has been associated with MACE, but whether it is solely a marker for these events or a mediator with the potential of representing a unique therapeutic target is uncertain. Our analysis set out to evaluate the current state of evidence linking PAI-1 antigen and activity levels with these outcomes. Our study suggests that elevated PAI-1 antigen levels are associated with major adverse cardiac events in both primary and secondary event populations. In addition, elevated PAI-1 antigen levels were associated with all-cause mortality. While the populations studied were heterogeneous, the robustness of the association suggests that PAI-1 warrants further study as a marker and potential mediator of adverse cardiovascular events.

Our findings build upon the growing evidence that PAI-1 is a biomarker for MACE in patients with CAD. Tofler *et al.* identified elevated PAI-1 antigen levels to be predictive of cardiovascular disease [178]. In addition, previous studies of elevated PAI-1 antigen and activity levels predicted acute coronary syndrome after coronary stenting [112, 154, 176, 179]. Furthermore, Song *et al.* recently identified a causal relationship between elevated PAI-1 levels and incident CAD [25]. Our study expands on these findings by identifying elevated PAI-1 antigen levels as being associated with MACE in both primary (incident) and secondary event populations thereby suggesting a broader relevance of PAI-1 concentration. In addition, we demonstrate the potential applicability of PAI-1 antigen levels in predicting restenosis, consistent with a previous report by Katsaros *et al.* [180], which identified that patients with the lowest PAI-1 antigen tertile had a 9.5-fold increased risk of in-stent restenosis in patients managed with modern drug-eluting stents. However, our study, as with those mentioned above, are unable to ascertain if PAI-1 is a mediator or simply a marker of these events. Further study is needed to establish this important distinction.

The association of PAI-1 activity with MACE did not meet our pre-specified thresholds for significance. Although this finding suggests that PAI-1 antigen levels may be more robust as a biomarker, PAI-1 activity is a functional measure of the entire PAI-1 content in the plasma. The measurement of PAI-

1 antigen captures the entire PAI-1 content in the sample in the form of free and active PAI-1 (which we refer to as PAI-1 activity), PAI-1 complexed to t-PA or u-PA, and latent PAI-1. Although PAI-1 antigen and activity levels are correlated, antigen levels will not necessarily reflect PAI-1 activity levels [54]. Indeed, at time of acute trauma such as plaque rupture, t-PA will complex with PAI-1 at a 1:1 ratio reducing detectable PAI-1 activity but not PAI-1 antigen levels. In addition, PAI-1 activity is influenced by experimental techniques during sample isolation such as freeze-thaw or sonication [39], low temperature, low pH, and high salt concentrations [56]. Factors which influence PAI-1 activity levels which impacted the significance of our findings include the method of PAI-1 extraction and isolation [39, 56, 90-92], time of blood draw [85], intra- and inter-assay variability in PAI-1 activity and antigen levels [89], and baseline risk factors which influences PAI-1 levels such as smoking [36, 86], high-fat diet [88], and maximal exercise [87]. Finally, in addition to important biological differences, manifest differences in the quality and power of studies examining antigen and activity levels existed which may explain the divergent results.

In-stent restenosis (ISR) is a result of neointimal formation or intimal thickening that narrows the vascular lumen following PCI [108]. The detailed molecular mechanism behind the pathophysiology of ISR has been reviewed elsewhere [109]. Briefly, studies have revealed that the initial recruitment of inflammatory cells is subsequently followed by smooth muscle cells (SMC) and myofibroblasts recruitment, which creates the extracellular matrix that narrows the vascular lumen [109]. SMCs achieve their peak proliferation at 48-96 hours post-injury in the media and intima and return to their baseline following re-endothelialization of that artery within 8 weeks [181]. Conflicting evidence exists in the literature in the role of PAI-1 in cell migration. PAI-1 binding to low-density lipoprotein receptor-related protein (LRP1) in SMCs promotes cell migration [61]. However, PAI-1 complexed to vitronectin has been demonstrated to inhibit cell migration and adhesion [75]. Thus, biological plausibility exists to link PAI-1 and restenosis following coronary intervention.

Clinically, low PAI-1 antigen and activity levels have been found to be associated with increased restenosis in our study; however, several limitations exist in these studies. First, these selected studies range from 1991 to 2008, during which time the intervention of choice evolved from balloon angioplasty to bare-metal stents to drug-eluting stents, which reduced the rate of ISR observed today [1]. Second, anti-proliferative agents that coat drug-eluting stents such as paclitaxel promote PAI-1 transcription and translation, impacting PAI-1 levels at the site of injury [124]. Third, PAI-1 activity cannot detect PAI-1 complexed to LRP1 found on smooth muscle cells and endothelial cells as they are no longer in circulation. Finally, since ISR occurs months following intervention, it remains unclear if baseline levels alone would be as predictive as repeated measurements. Repeat measurements of PAI-1 levels in these patients which would provide a comprehensive assessment of temporal PAI-1 levels from baseline to follow-up angiography or re-intervention. Nonetheless, despite these limitations we were able to link basal PAI-1 levels and restenosis. Future studies looking at modern revascularization techniques and temporal patterns of PAI-1 are warranted.

The value of PAI-1 as a biomarker has been questioned. First, PAI-1 expression is influenced by multiple pro-inflammatory conditions and is associated with various cardiovascular risk factors [134, 182]. For example, metabolic syndrome, obesity [32] and hyperinsulinemia [136]/insulin resistance [83] have all been linked with increased PAI-1 levels. In adjusted analysis, the predictive ability of elevated PAI-1 levels has not been independent of other cardiovascular risk factors and its additive benefit in risk prediction models has been lacking [80]. For example, Yarmolinsky *et al.* [48] reported that patients with diabetes had a significantly higher level of plasma PAI-1, which was associated with MACE. However, diabetics are at increased risk of both index and recurrent events. Accordingly, further studies are needed in more homogenous populations to ascertain the performance of PAI-1 in each individual cohort.

Our study is not without limitations. Relevant data could not be obtained from certain studies and patient level data were not available. In addition, the broad inclusion criteria resulted in a heterogeneous study population with differing PAI-1 measurement techniques. Variations in assays and standardizations

as well as natural variations in PAI-1 levels may have influenced our results. For instance, considerable PAI-1 diurnal changes have been observed in previous studies [183, 184]. Most selected studies were of modest sample size and of low or moderate quality with only seven studies deemed to be of high quality [185]. The small number of studies may have limited the detection of small study effects or publication bias in funnel plots [186]. Finally, the large variation in study dates (1991 to 2016) spans a broad range of pharmacologic and revascularization practices, particularly coronary stent development, the introduction of dual antiplatelet therapy, and broadening indications for oral anticoagulation therapy. Accordingly, these findings may not be applicable in patients with specific risk profiles or those on contemporary medical therapy. Finally, while our study is provocative in the association demonstrated interventional studies are needed to link PAI-1 levels mechanistically to MACE.

2.7 Conclusion

PAI-1 plasma levels are promising markers for MACE; however high-quality studies in well-defined populations are still needed to robustly evaluate the performance of PAI-1 as a clinical biomarker. Whether PAI-1 is a bona fide therapeutic target remains to be established.

2.8 Sources of Funding

R.G.J. was supported by the Vanier CIHR Canada Graduate Scholarship for his graduate studies.

2.9 Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest for the completion of the manuscript.

Chapter 3

Performance of plasminogen activator inhibitor-1 as a biomarker in patients undergoing coronary angiography: analytical and biological considerations

3.1 Preface

This chapter has been previously published as an article in *Diabetes and Vascular Disease Research*:

> **Jung RG**, Simard T, Di Santo P, Labinaz A, Moreland R, Duchez AC, Majeed K, Motazedian P, Rochman R, Jung Y, Hibbert B. Performance of plasminogen activator inhibitor-1 as a biomarker in patients undergoing coronary angiography: Analytical and biological considerations. *Diab Vasc Dis Res*. 2019;16:478-482

The result and discussion from the published version of the manuscript was separated into its respective sections.

3.2 Abstract

Introduction and Objective: Target lesion failure continues to limit the efficacy of percutaneous coronary intervention despite advancements in stent design and medical therapy. Identification of biomarkers to risk stratify patients post-PCI has the potential to focus therapies on cohorts with increased benefits. Plasminogen activator inhibitor-1 (PAI-1) has been identified as a candidate biomarker. Herein, we evaluate biological variables which impact PAI-1 levels and analytical characteristics which impact its utility as a biomarker in humans.

Methods: Plasma PAI-1 was measured in 689 patients undergoing coronary angiography. PAI-1 levels were measured. Clinical and procedural characteristics were collected in a prospective registry.

Results: Plasma PAI-1 analytical (CV_a = 4.1%), intra-individual (CV_i = 44.0%), and inter-individual (CV_g = 118.6%) variations with reference change value (RCV) of 61.8% were calculated. PAI-1 levels were

elevated in patients with cardiovascular risk factors including type 2 diabetes, pre-diabetes, smokers, obesity, hypertension, and daytime variation in procedure and blood draw.

Conclusion: Variation in plasma PAI-1 levels is influenced by multiple biological and procedural characteristics. The performance of plasma PAI-1 is consistent with biomarkers in clinical use (NT-proBNP and C-reactive protein) and its applicability is promising.

3.3 Introduction

Plasminogen activator inhibitor-1 (PAI-1) is an anti-fibrinolytic protein involved in the regulation of fibrin clot formation.[187] By forming an inhibitory irreversible 1:1 complex with tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), PAI-1 prevents fibrin clot degradation promoting hemostasis.[187] Although PAI-1 is expressed in multiple cell types, circulating plasma PAI-1 is primarily platelet-derived.[188] Mechanisms responsible for elevated plasma PAI-1 levels remain unknown; however, increased levels have been associated with type 2 diabetes, metabolic syndrome, and coronary artery disease.[48, 189]

Previous small studies have evaluated PAI-1 as a biomarker - quantifying plasma PAI-1 antigen as opposed to activity levels. While PAI-1 is linked to vascular risk factors, its function as a biomarker to predict cardiovascular adverse events has yielded mixed results due to heterogeneity in the populations studied and methodologies used. Moreover, PAI-1 levels may be influenced by technical factors such as time of blood draw and method of extraction.[85, 90] Finally, analytic characteristics of PAI-1 have yet to be reported.[190]

Accordingly, we sought to determine the performance of PAI-1 following percutaneous coronary intervention (PCI). In particular, PAI-1 may play roles in thrombotic events and neointimal hyperplasia following revascularization.[187] Given the lack of robust data evaluating plasma PAI-1 as a biomarker in this population, we set out to determine which patient, clinical, and procedural factors influence PAI-1 antigen

levels to inform future outcome-based studies. Herein, we report biological and analytical considerations in PAI-1 measurement in patients undergoing coronary angiography and revascularization.

3.4 Methods

3.4.1 Ethics Statement

This study was approved by the Ottawa Health Science Network Research Ethics Board (OHSN-REB) (Protocol #: 20160516-01H) and written informed consent was obtained from all patients. The study conforms with the 1975 Declaration of Helsinki for the use of human blood.

3.4.2 Study Design and Patient Data Collection

The University of Ottawa Heart Institute is a quaternary care center servicing a catchment of 1.2 million people with all procedures prospectively indexed in a registry. From October 2016 to August 2018, blood samples were collected on consecutive eligible patients referred for PCI**.** Arterial blood samples were collected in 3.2% sodium citrate tubes (Becton Dickinson, Franklin Lakes, NJ, USA) immediately after the completion of the procedure and processed within two hours after collection. Samples were centrifuged at 1,200 x g for 10 minutes at 4°C without brakes. Next, platelet-rich plasma was spun at 3,200 x g for 10 minutes at 4°C to remove contaminants and the plasma were stored at -80°C until further processing.

Patient data was obtained from the Cardiovascular And Percutaneous Clinical TriALs (CAPITAL) revascularization registry which indexes over 1,200 clinical data points on patients undergoing coronary angiography at the institute. Coronary artery disease confirmed by coronary angiography was defined as stenosis ≥ 50%. Time of procedure was collected and dichotomized to morning (800 – 1200 hours) or afternoon (1200 – 1600 hours). Diabetes status was defined as hemoglobin A1c (HbA1c) levels ≥6.5% at presentation. Prediabetes was defined as having HbA1c levels between 6.0-6.4% according to the Canadian Diabetes Association guidelines. Medication status was indexed at the time of the procedure.

3.4.3 PAI-1 Quantification

Plasma samples from 689 patients were thawed and PAI-1 antigen levels were determined by a commercially available enzyme-linked immunosorbent assay (ELISA) according to the manufacturers' recommendations (KHC3071) (ThermoScientific, Waltham, Massachusetts, USA). Laboratory assessment of PAI-1 antigen levels from patient samples were performed within 6 months of storage by the same research personnel to minimize analytical variations.

3.4.4 Statistical Analysis

Continuous variables were reported with mean ($\pm$ standard deviation) or median (interquartile range) as appropriate. Categorical variables were reported as proportions (%). Plasma PAI-1 levels was first examined for normality using Shapiro-Wilks test, and subsequently, standard t-test or Mann-Whitney U-test was performed as appropriate. Coefficient of variation (CV) was determined for analytical coefficient of variability (CV_a), inter-subject CV in our population (CV_g), and intra-subject CV (CV_i).[190, 191] Index of individuality (II) allows the determination of whether monitoring or reference range is required for a biomarker.

$$CV = \frac{\sigma}{\mu}\ x\ 100\%$$

$$CV_i = (CV_t^2 - CV_a^2)^{\frac{1}{2}}$$

$$Index\ of\ individuality\ (II) = \frac{\left(CV_a^2 + CV_i^2\right)^{\frac{1}{2}}}{CV_g}$$

$$Reference\ change\ value\ at\ P \leq 0.05 = \ 2.77 * \ \left(CV_a^2 + CV_i^2\right)^{\frac{1}{2}}$$

Univariate linear regression was performed with all factors with a predetermined p-value < 0.20 to identify variables for inclusion prior to a multivariable linear regression analysis. All statistical analyses were performed using SAS v9.4 (SAS Institute, Inc.). P-value < 0.05 was considered significant. Figures were created using GraphPad Prism v7 (GraphPad Software, La Jolla, CA, USA).

3.5 Results

3.5.1 Baseline Characteristics

PAI-1 antigen levels were measured in 689 patients. The mean age of our cohort at baseline was 67.3 $\pm$ 11.2 years, and 66.9% were male (Table 3-1). Overall, our cohort was overweight with an average body mass index of 29.6 $\pm$ 6.5 kg/m^2. Cardiac risk factors were prevalent including pre-diabetes (9%), type 2 diabetes (37%) of which 29.5% were insulin-managed, active smoker (34%), dyslipidemia (63%), and hypertension (65%). Patients were managed with contemporary medical therapy with most patients receiving statins (78%), ASA (89%), and $P2Y_{12}$ inhibitors (89%). A high proportion of patients had obstructive coronary artery disease (79%). A total of 46% of patients underwent PCI, with 89% performed via trans-radial access and 86% having received unfractionated heparin during the procedure.

Table 3-1. Baseline characteristics of patients undergoing coronary angiography with measured plasma PAI-1 levels

	Plasma PAI-1 levels (pg/mL) (n = 689)	
	Number or mean	Proportion (%) or Standard Deviation
Age (mean +/- SD)	67.3	11.2
Male (%)	461	66.9
Height (m)	1.7	0.1
Weight (kg)	85.9	20.3
Body mass index (kg/m2)	29.5	6.2
Biochemistry		
Hemoglobin	133.4	18.4
Platelet count	220.9	70.6
Mean platelet volume	10.5	1.0
Creatinine clearance (mL/min)	103.7	49.6
HbA1c (%)	6.4	1.3
Indication for angiography		
ACS	273	39.6
Stable CAD	272	39.6
Staged PCI	63	9.2
Shock	1	0.2
Arrhythmia	9	1.3
Heart failure/LV Dysfunction	54	7.9
Cardiac risk factors		
Diabetes		
Type I	3	0.4
Type II	254	36.9
Smoking	236	34.3
Dyslipidemia	433	62.8
Family history	110	16.0
Hypertension	450	65.3
Prior history		
CAD	235	34.1
PCI	164	23.8
MI	124	18.0
CABG	36	5.2
CVA/TIA	42	6.1
Medications		
ACEi/ARB	366	53.1
Beta blocker	407	59.1
Calcium channel blocker	105	15.2
Statin	535	77.7
Oral anticoagulation	46	6.7
IV unfractionated heparin	77	11.2
Subcutaneous LMWH	78	11.3
ASA	615	89.3
P2Y12	611	88.7
Loading dose within 24hrs	362	55.7
Procedural Access		
Radial	608	89.4
Femoral	70	10.3
Brachial	2	0.3
Access site medications		
CCB	363	53.4
Nitroglycerin	224	32.9
Procedural medications		
Heparin	584	85.9
Heparin Dose	6195.4	2751.6
Adenosine	43	6.3
Nitroglycerin	267	39.3
Number of cases with (>50%) CAD	541	78.5
PCI conducted	315	45.7

ACEi/ARB = Angiotensin-converting enzyme inhibitor/aldosterone II receptor blocker
ASA = acetylsalicylic acid
CCB = calcium channel blocker
CVA/TIA = cerebrovascular accident/transient ischemic attack
LMWH = low molecular weight heparin

3.5.2 Analytical Performance of Plasma PAI-1

To assess biomarker performance, we assessed CVs in our population of interest. Since plasma PAI-1 levels were not normally distributed, CVs are reported for both baseline and log-transformed data. The analytic coefficient of variation for the assay (CV_a) was low (n = 80) at 4.1%. Variability within subjects (intra-subject coefficient of variation - CV_i) of those with a repeat sample in one year was moderate at 44.0% (n = 40). Variability between subjects (inter-subject coefficient of variation - CV_g) in our entire cohort shows wide variation CV_g = 118.6% (n = 689). Finally, the index of individuality and reference change value (RCV) of PAI-1 were 0.4 and 61.8%, respectively. The CV_a, CV_i, and CV_g of the log transformed antigen levels were 0.7%, 24.8% and 12.9%, respectively. The performance of plasma PAI-1 antigen parallel established markers for coronary artery disease (CAD) such as C-reactive protein (CRP, CV_a = 5.2%, CV_i = 42.2%, CV_g = 92.5%, II = 0.5) and for heart failure such as NT-proBNP (CV_a = 3.3%, CV_i = 25.1%, CV_g = 54.0%, II = 0.2).[192, 193]

3.5.3 Impact of Biological Variables on Plasma PAI-1 Levels

Next, we sought to identify patient comorbidities, medications, and biochemical parameters which may have impacted PAI-1 levels. We first performed a univariate regression model and included all associated factors with p-value < 0.20 in a multivariable linear regression to identify associated variables (Table 3-2). We identified body mass index, active smoking, diabetes, pre-diabetes, and hypertension to be positively associated with changes in plasma PAI-1 levels (Figure 3-1A-C). Notably, confirmed presence of CAD did not influence PAI-1 antigen levels. New procedural factors were identified which potentially affect measured plasma PAI-1 levels such as the use of nitroglycerin and daytime variation in coronary angiography (Figure 3-1D-E). Finally, females appear to have higher circulating levels of PAI-1 (38.5% change) compared to their male counterparts (Figure 3-1F).

Table 3-2. Multivariable linear regression to determine variables associated with plasminogen activator inhibitor-1

Predictor	Parameter Estimate	Percentage Change in PAI-1 levels	95% Confidence Interval		P-value	p<0.05
Multivariable						
Time of procedure	-0.482	-38.3	-0.623	-0.341	<0.0001	**
Intra-arterial nitroglycerin	-0.217	-19.5	-0.368	-0.066	0.01	*
Age	-0.009	-0.9	-0.016	-0.001	0.02	*
Hemoglobin	-0.003	-0.3	-0.007	0.001	0.18	
Platelet count	0.000	0.0	-0.001	0.001	0.72	
Dyslipidemia	0.011	1.1	-0.145	0.167	0.89	
Body mass index	0.026	2.6	0.014	0.038	<0.0001	**
P2Y12	0.036	3.6	-0.205	0.277	0.77	
Intra-arterial heparin	0.046	4.7	-0.166	0.258	0.67	
Coronary artery disease	0.064	6.6	-0.121	0.248	0.50	
Statin	0.142	15.3	-0.049	0.334	0.15	
Hypertension	0.170	18.5	0.008	0.331	0.04	*
Diabetes	0.219	24.5	0.055	0.383	0.01	*
Gender	0.291	33.7	0.131	0.450	<0.001	**
Pre-diabetes	0.300	35.0	0.074	0.526	0.01	*
Smoking	0.317	37.3	0.167	0.468	<0.0001	**
R-squared	0.239					
Adjusted R-squared	0.217					
Total number of observations	551					

*P<0.05

**P<0.0001

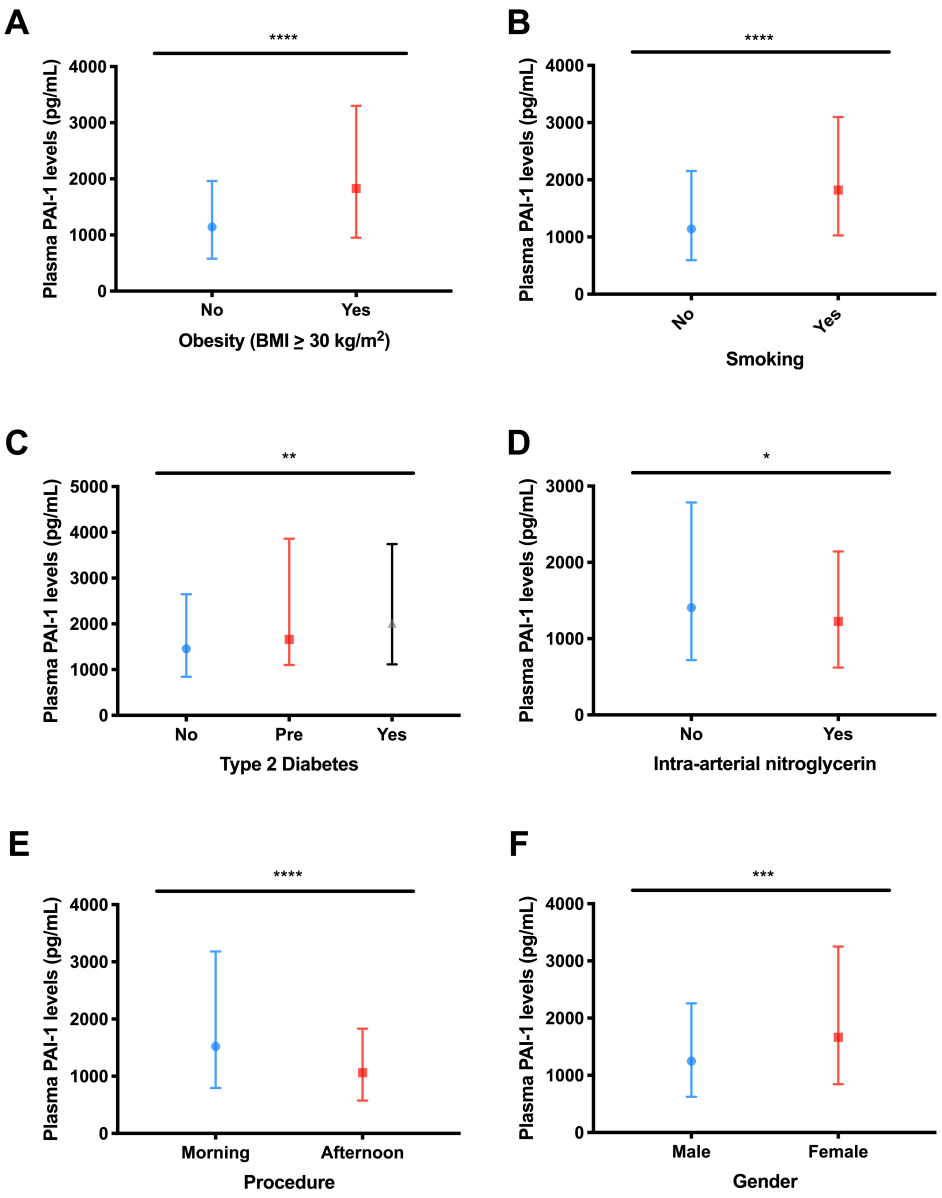


Figure 3-1. Influence of plasma PAI-1 levels (pg/mL) by biological and procedural characteristics.

(**A**) PAI-1 levels are elevated with obesity (BMI ≥ 30 kg/m^2) (1828.0 [951.6 – 3301.0] pg/mL (n = 272) vs. 1140.0 [577.0 – 1961.0] pg/mL (n = 417), p<0.0001). (**B**) PAI-1 levels are elevated with active smoking (1822.0 [1028.0 – 3100.0] pg/mL (n = 236) vs. 1143.0 [596.6 – 2156.0] pg/mL (n = 453), P<0.0001). (**C**) PAI-1 levels are elevated with type 2 diabetes mellitus (2014.0 [1115.0 – 3743.0] pg/mL (n = 210) vs. 1661.0 [1103.0 – 3861.0] pg/mL (n = 62) vs. 1455.0 [842.3 – 2647.0] pg/mL (n = 181), p<0.01). (**D**) PAI-1 levels are decreased with intra-arterial administration of nitroglycerin (1227.0 [620.9 – 2144.0] pg/mL (n = 267) vs. 1407.0 [719.2 – 2785.0] pg/mL (n = 413), p=0.03). (**E**) PAI-1 levels are decreased in procedures performed in the afternoon (1063.0 [576.6 – 1832.0] pg/mL (n = 270) vs. 1521.0 [796.5 – 3183.0] pg/mL (n = 406), p<0.0001). (**F**) PAI-1 levels are elevated in females (1665.0 [846.6 – 3252.0] pg/mL (n = 225) vs. 1249.0 [624.0 – 2258.0] pg/mL (n = 460), p<0.0001). Data is presented as median ± interquartile range. Mann-Whitney U-test and Kruskal-Wallis test was conducted for non-parametric data. *P<0.05. **P<0.01. ***P<0.001. ****P<0.0001.

3.6 Discussion

Herein, we demonstrate that plasma PAI-1 has similar analytical performance characteristics to clinically used biomarkers such as CRP and NT-proBNP which have high CV_g and CV_i. Due to a low index of individuality of PAI-1 (II <0.6), changes from population-based reference values in an individual have limited value as a diagnostic test to detect pathologies but may be useful as a monitoring test in patients undergoing coronary angiography.[190, 194] The measured critical difference (at $P \leq 0.05$) between two serial measurements of PAI-1 was determined to be 122%, similar in performance to CRP (118%).[192] Thus, use of PAI-1 will be of little benefit in screening healthy populations for early stages of CAD but may be useful when serial monitoring is performed to examine response to therapy or ascertaining risk.[180]

To date, our report is derived from the largest cohort of patients undergoing coronary angiography and the first to report the analytical performance of PAI-1 as a biomarker. Our study confirms known factors impacting PAI-1 levels while also highlighting factors including circadian variation, female sex, and pre-diabetes – factors which should be incorporated into future studies.[195] Whether plasma PAI-1 levels predict outcomes following coronary revascularization remains to be established in larger cohorts.

Similar levels of plasma PAI-1 levels were observed in those with pre-diabetes and type 2 diabetes in those undergoing coronary angiography. This likely is due to two reasons contributing to the pro-inflammatory state observed in diabetes: 1) pre-diabetes is often not found exclusively in patients presenting for a coronary angiogram and is often found in concert with other cardiovascular risk factors including hypertension, dyslipidemia, and obesity forming the foundation of the diagnostic criteria for metabolic syndrome; [196, 197] and 2) platelet hyper-reactivity is observed in pre-diabetes exhibited by increase in platelet aggregation and potentially, subsequent release of PAI-1 from α-granules. [68, 188, 198]

Our study is not without limitations. First, variation of circulating PAI-1 levels due to circadian rhythm cannot be fully captured by accounting for the daytime variation of coronary angiogram since each individual has a variable diurnal pattern.[85] Second, this remains a retrospective analysis although the registry data is collected prospectively and independently of PAI-1 samples to minimize bias. Finally, this

study remains underpowered to link PAI-1 levels with clinical outcomes, and although a large sample size was achieved given the observed CV_i and CV_g, future studies will need to enrol significantly larger cohorts to assess this relationship.

3.7 Conclusion

In summary, plasma PAI-1 antigen levels demonstrate similar biomarker performance characteristics to established clinically utilized biomarkers. Pre-diabetes, diabetes, sex, and smoking status are positively associated with PAI-1 levels. In contrast, age, procedural administration of intra-arterial nitroglycerin, and daytime variation in coronary angiogram appear to be inversely associated with PAI-1 levels. Careful consideration of biological and analytical variations should be incorporated into future studies.

3.8 Sources of Funding

This project was supported by the Innovation Fund of the Alternative Funding Plan for the Academic Health Sciences Centres of Ontario and Canadian Foundation of Innovation. R.G.J. was funded by the Vanier CIHR Canada Graduate Scholarship for his graduate studies. The authors would like to thank the CAPITAL Research Group for all their assistance in the completion of the project.

3.9 Conflict of Interest

The authors do not have any reported conflicts of interest.

Chapter 4

Evaluation of plasminogen activator inhibitor-1 as a biomarker of unplanned revascularization and major adverse cardiac events in coronary angiography and percutaneous coronary intervention

4.1 Preface

This chapter has been previously published as an article in *Thrombosis Research*:

> **Jung RG**, Simard T, Di Santo P, Dhaliwal S, Sypkes C, Duchez A-C, Moreland R, Taylor K, Parlow S, Visintini S, Labinaz A, Marbach J, Sarathy K, Bernick J, Joseph J, Boland P, Abdel-Razek O, Harnett DT, Ramirez FD, Hibbert B. Evaluation of plasminogen activator inhibitor-1 as a biomarker of unplanned revascularization and major adverse cardiac events in coronary angiography and percutaneous coronary intervention. *Thrombosis Research*. 2020

4.2 Abstract

Background: The stented coronary artery remains at risk of complications, particularly in the form of stent thrombosis and in-stent restenosis. Improving our ability to identify patients at high-risk for these complications may provide opportunities for intervention. PAI-1 has been implicated in the pathophysiology of stent complications in preclinical studies, suggesting it may be a clinically valuable biomarker to predict adverse events following percutaneous coronary interventions (PCI).

Methods: Plasma PAI-1 levels were measured in 910 subjects immediately after coronary angiography between 2015-2019. The primary outcome was the incidence of unplanned revascularization (UR) at 12 months. The secondary outcome was the incidence of major adverse cardiac events (MACE).

Results: UR and MACE occurred in 49 and 103 patients in 12 months. Reduced plasma PAI-1 levels were associated with UR (4386.1 pg/mL [IQR, 2778.7-6664.6], n=49, vs. 5247.6 pg/mL [IQR, 3414.1-7836.1], n=861; p=0.04). Tertile PAI-1 levels were predictive of UR after adjustment for known clinical risk factors associated with adverse outcomes. In *post-hoc* landmark analysis, UR was enhanced with low plasma PAI-1 levels for late complications (beyond 30 days). Finally, an updated systematic review and meta-analysis did not reveal an association between plasma PAI-1 and MACE.

Conclusion: PAI-1 levels are not independently associated with UR nor MACE in patients undergoing angiography but associated with UR following adjustment with known clinical factors. In our landmark analysis, low PAI-1 levels were associated with UR for late stent complications. As such, future studies should focus on the mediatory role of PAI-1 in the pathogenesis of stent complications.

4.3 Introduction

Obstructive coronary artery disease (**CAD**) is often managed by percutaneous coronary intervention (**PCI**) and medical therapy.[1] Despite the clinical benefits of PCI, the stented coronary artery remains the highest risk lesion, with annualized event rates as high as 8-12% for death, myocardial infarction (MI), or stroke.[114, 118, 199] Two major complications following stent implantation are stent thrombosis (primarily an early thrombotic event) and in-stent restenosis (primarily a late process of intimal hyperplasia).[108, 200] Although patient-, stent-, and procedure-related risk factors for stent complications have been identified, no biomarker yet exists for risk stratification following PCI.

Numerous studies have examined plasma plasminogen activator inhibitor-1 (**PAI-1**) as a biomarker of obstructive CAD patients with no prior history of CAD;[201, 202] however, little is known about the predictive ability of PAI-1 following the diagnosis of significant CAD. PAI-1 is a critical inhibitor of the fibrinolysis pathway, which functions by complexing with tissue-type plasminogen activator (**tPA**) to prevent fibrin degradation, thus potentially increasing a patient's thrombotic risk.[203, 204] In the post-PCI population, this potentially manifests early as stent thrombosis, typically in the first 30 days. Indeed, elevated levels of plasma PAI-1 have been associated with major adverse cardiac events (**MACE** – composite of death, myocardial infarction, unplanned revascularization, and stroke) in all-comers, as previously described by our group.[205] PAI-1 is also a regulator of smooth muscle cell (**SMC**) proliferation and has been linked to intimal hyperplasia following vascular injury. After PCI, this may manifest as in-stent restenosis (**ISR**) – a process that occurs 1-12 months after stent implantation which results in late lumen loss. Although PAI-1 has been implicated in the pathophysiology of both ISR and stent thrombosis, its role as a potential biomarker for these events is unknown.

Given the potential role of PAI-1 pathophysiology to mediate stent complications and its promising analytical and biochemical performance in humans,[206] we sought to examine the association of plasma PAI-1 with unplanned revascularization (**UR**) and MACE following coronary angiography and PCI.

4.4 Methods

4.4.1 Ethics Statement

This study was approved by the Ottawa Health Science Network Research Ethics Board (OHSN-REB Protocol #: 20160516-01H) and informed consent was obtained from all patients.

The study conforms with the 1975 Declaration of Helsinki for the use of human blood. The study was also approved by OHSN-REB (#20190224-01H) to evaluate clinical outcomes following revascularization.

4.4.2 Study Population and Data Collection

The University of Ottawa Heart Institute is a large tertiary center serving over 1.2 million people in Eastern Ontario. All coronary catheterization procedures performed in the region are registered in the Cardiovascular And Percutaneous clInical TriALs (CAPITAL) revascularization registry. This registry captures over 1,200 clinical data points on patient and procedural parameters, as previously described.[207] The presence of CAD was defined as ≥50% stenosis of an epicardial coronary artery on angiography. Time of procedure was dichotomized as morning (8:00-12:00 h) or afternoon (12:00-16:00 h). Patients undergoing coronary angiography or PCI in the evening were not included for workflow reasons.

4.4.3 Plasma Processing for Measurement of Plasma PAI-1 Levels

Arterial blood samples were collected from consecutive eligible patients referred for PCI between August 2015 and October 2019. Blood was collected in 3.2% sodium citrate tubes (Becton Dickinson, Franklin Lakes, NJ, USA) immediately upon the completion of the procedure and processed within two hours. Samples were centrifuged at 1,200 x g for 10 minutes at 4°C without brakes then spun at 3,200 x g for 10 minutes to remove further contaminants before being stored at -80°C until further processing. Plasma was analyzed in a 1:5 dilution using human PAI-1 enzyme-linked immunosorbent assay (ab184863, Abcam, Cambridge, United Kingdom) as per manufacturer's recommendations by a researcher blinded to the patient and procedural details.

4.4.4 Follow-up and Outcomes

Outpatient clinic or chart review follow-up was completed to evaluate the endpoints of UR and major adverse cardiac events. UR occurred when patients unexpectedly returned to the catheterization lab for coronary revascularization. Clinicians and personnel who conducted the clinical follow-up to determine adverse outcomes by a clinic visit or chart review were blinded to plasma PAI-1 levels. Myocardial infarction and UR events were captured upon patients returning to the Ottawa Heart Institute. Clinical characteristics at one-year follow-up was captured and recorded through the CAPITAL Revascularization Registry.

4.4.5 Systematic Review Search Strategy

An update was performed of a previously described systematic review and meta-analysis.[205] The updated literature search was performed by a medical librarian (S.V.) on December 18, 2019 using a combination of key terms related to PAI-1 as previously described using date limiters (see Table 4-1).[205] Title and abstracts of all eligible studies were screened by two independent reviewers using Covidence (Level 10, Melbourne, Australia) and selected manuscripts for full review were independently reviewed for inclusion. Inclusion criteria, quality assessment, and data extraction methods were all consistent with our previous description.[205] However, exclusion criteria remained similar as the previous manuscript with the addition of the following criteria for exclusion: (1) studies examining the relationship of PAI-1 activity with outcomes, (2) angiography-determined restenosis as the primary outcome, and (3) studies where fibrinolytics were utilized as the primary revascularization method. Finally, study quality was assessed using the Newcastle-Ottawa Scale.

Table 4-1. Search Strategy for Ovid MEDLINE (<1946 to Dec 17, 2019

#	Search Strategy	Results, n
1	exp Myocardial Ischemia/	422285
2	(myocard* adj5 (isch?em* or infarct*)).tw.	224707
3	ami.tw.	18426
4	(heart adj5 infarct*).tw.	15222
5	(isch?emi* adj5 heart).tw.	48072
6	(coronary adj5 (stenosis or restenosis)).tw.	17491
7	exp Coronary Artery Bypass/	51987
8	cabg.tw.	17352
9	CAG.tw.	9941
10	angina?.tw.	53627
11	(coronary adj5 (disease* or bypass or thrombo* or arter?oscleros*)).tw.	191751
12	chd.tw.	24052
13	acute coronary syndrom*.tw.	29612
14	acs.tw.	20663
15	exp Percutaneous Coronary Intervention/	51684
16	(percutaneous coronary adj2 (interven* or revascular*)).tw.	31890
17	pci.tw.	24181
18	ptca.tw.	6458
19	exp Angioplasty/	60709
20	angioplast*.tw.	42692
21	((coronary or arterial) adj4 dilat*).tw.	5381
22	endoluminal repair*.tw.	215
23	exp Stents/	75004
24	stent*.tw.	97017
25	exp Atherectomy/	2416
26	atherectom*.tw.	2975
27	or/1-26	716805
28	Plasminogen Activator Inhibitor 1/	8570
29	PAI-1.tw.	10510
30	Plasminogen activator inhibitor-1.tw.	7791
31	SERPIN*.tw.	5949
32	or/28-31	19889
33	27 and 32	1748
34	Animals/	6530212
35	Humans/	18185496
36	34 not 35	4622731
37	33 not 36	1616
38	limit 37 to ed=20161001-20201230	85
39	limit 37 to dt=20161001-20201230	93
40	38 or 39	118

4.4.6 Statistical Analysis

Continuous variables were reported as mean ± standard deviation or median ± interquartile range as appropriate. Categorical variables were reported as proportions (%). Median plasma PAI-1 levels were compared using Mann-Whitney U-tests. The primary outcome for this study was to determine the predictive ability of plasma PAI-1 and unplanned revascularization. Secondary outcomes of interest were to determine the relationship between plasma PAI-1 with MACE and individual components of MACE.

Plasma PAI-1 levels were categorized into tertiles (30^{th} and 70^{th} percentiles) and Kaplan-Meier curves were generated to evaluate time-to-event distributions and compared by log-rank tests. Hazard ratios (**HR**) and corresponding 95% confidence intervals were obtained from Cox proportional-hazard models after testing for proportionality via the martingale residual method.[208] Multivariable Cox proportional-hazards model including predetermined variables were fit to evaluate the association of PAI-1 and established risk factors with the risk of UR and MACE. *Post-hoc* landmark analysis from 0 to 30 days for early complications (during the peri-procedural period) and from 30 days to one year for late complications (major risk for in-stent restenosis) were evaluated for both UR and MACE.[209]

A meta-analysis of the association between plasma PAI-1 levels (pg/mL) and MACE was conducted using Review Manager 5.3 (RevMan, Cochrane Collection, Copenhagen, Denmark). Mean differences in plasma PAI-1 levels were compared between event and no-event groups using random effect models using the inverse variance method. All effect estimates are reported as mean differences with 95% confidence intervals.

All statistical analyses were performed using SAS v9.4 (SAS Institute, Inc., Cary, NC, USA). $P<0.05$ was considered statistically significant. Forest plots were generated using RevMan

and all remaining figures were created using GraphPad Prism v8 (GraphPad Software, La Jolla, CA, USA).

4.5 Results

The study flow for evaluating plasma PAI-1 as biomarker of adverse events following coronary angiography and/or PCI is depicted in Figure 4-1. A total of 1851 patients underwent cardiac catheterization and had blood samples collected between August 2015 and October 2019 at our center, with a total of 1125 clinical follow-ups completed. During that period, 215 samples were excluded as they were exhausted for other studies or due to poor processing of samples, and 910 plasma PAI-1 samples were measured. The mean age was 66.4 $\pm$ 11.6 years and females accounted for 25.7% of the cohort. The mean body mass index was 29.1 $\pm$ 5.6 kg/m^2. Co-morbidities included type 2 diabetes mellitus (25.5%), active smoking (16.6%), dyslipidemia (61.5%), and hypertension (62.5%). Furthermore, indications for angiography varied with 42.1% presenting with acute coronary syndrome, 36.9% with stable CAD, and 13.8% with staged PCI. Ultimately, 63.7% of the cohort underwent PCI or CABG following coronary angiography (Table 4-2).

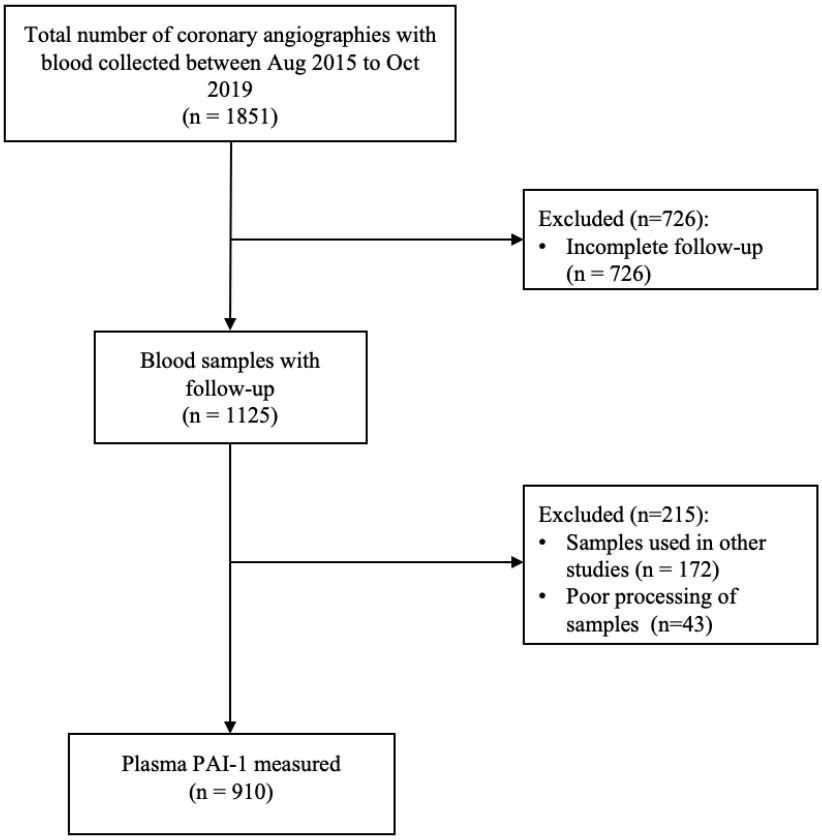


Figure 4-1. Flow diagram for patient selection and plasma PAI-1 quantification

Table 4-2. Baseline characteristics

	Total (n=910)
Age - mean +/- SD	66.4 ± 11.6
Sex (female) - no. (%)	234 (25.7)
BMI - mean +/- SD	29.1 ± 5.6
Hypertension - no. (%)	569 (62.5)
Dyslipidemia - no. (%)	560 (61.5)
Type II diabetes - no. (%)	232 (25.5)
Diet	15 (1.6)
Oral hypoglycemic agents	149 (16.4)
Insulin	68 (7.5)
HbA1c - mean ± SD %	5.4 ± 2.4
Smoking - no. (%)	
Never	509 (55.9)
Remote (quit >1 month ago)	250 (27.5)
Active	151 (16.6)
Family history of CAD - no. (%)	143 (15.7)
Atrial fibrillation - no. (%)	85 (9.3)
Indication for angiography – no. (%)	
Acute coronary syndrome	383 (42.1)
Staged PCI	126 (13.8)
Stable CAD	336 (36.9)
Shock	1 (0.1)
ROSC	3 (0.3)
Previous History - no. (%)	
PCI	259 (28.5)
MI	215 (23.6)
CABG	51 (5.6)
PAD	58 (6.4)
CVA	49 (5.4)
Bleed	14 (1.5)
Heart failure	57 (6.3)
Medications - no. (%)	
ASA	839 (92.2)
P2Y12	840 (92.3)
ACEi/ARB	503 (55.3)
Beta blocker	569 (62.5)
Calcium channel blocker	115 (12.6)
Statin	755 (83.0)
PPI	171 (18.8)
NSAID	10 (1.1)
Time of procedure - no. (%)	413 (45.4)
Revascularized (stent + CABG) - no. (%)	580 (63.7)

4.5.1 Differences in Plasma PAI-1 Levels Following Coronary Angiography

Patients who underwent unplanned revascularization had lower levels of plasma PAI-1 levels (4386.3 pg/mL [2778.7-6664.6], n=49 vs. 5247.6 pg/mL [3414.1-7836.1], n=861; p=0.04) (Figure 4-2A). Plasma PAI-1 levels were similar between patients with and without MACE (5054.7 pg/mL [2967.6-7221.0], n=103 vs. 5243.3 pg/mL [3414.1-7836.1], n=807; p=0.33) (Figure 4-2B). Similarly, no differences in PAI-1 levels were seen in those with and without MI, UR, and stroke (4917.8 pg/mL [2892.1-7186.5], n=76 vs. 5245.4 pg/mL [3414.1-7836.1], n=834; p=0.13) (Figure 4-2C), death (5987.9 pg/mL [3363.6-7570.1], n=37 vs. 5225.8 pg/mL [3381.6-7687.3], n=873; p=0.74) (Figure 4-2D), MI (6426.6 pg/mL [3333.1-7411.5], n=19 vs. 5222.6 pg/mL [3363.6-7727.4], n=891; p=0.72) (Figure 4-2E), or stroke (6280.9 pg/mL [2960.4-7490.0], n=17 vs. 5225.8 pg/mL [3381.6-7687.3], n=893; p=0.97] (Figure 4-2F).

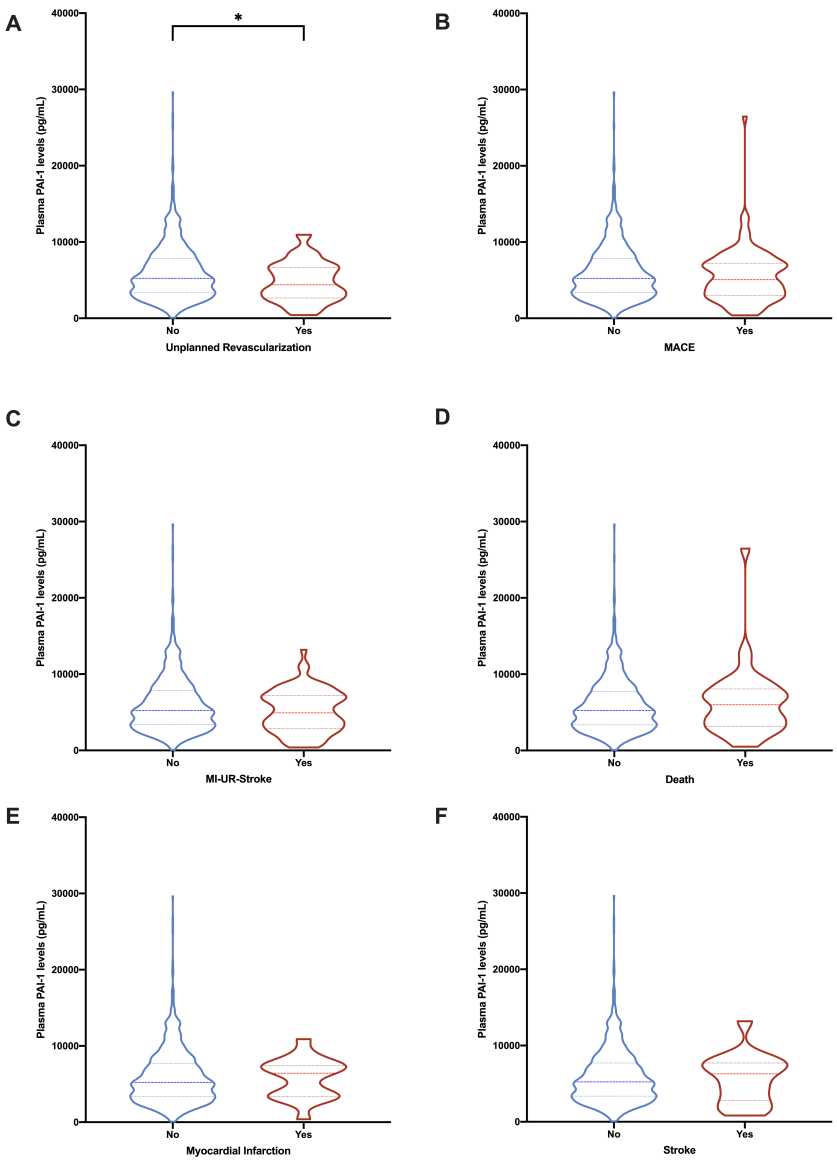


Figure 4-2. Difference in absolute plasma PAI-1 levels (pg/mL) by clinical outcomes.

(**A**) PAI-1 levels were reduced in those with unplanned revascularization (4386 pg/mL [2659-6670], n=49 vs. 5248 pg/mL [3414-7852], n=861; p=0.04). (**B**) Plasma PAI-1 levels were not different between patients with and without MACE (5055 pg/mL [2968-7221], n=103 vs. 5243 pg/mL [3414-7836], n=807; p=0.33). (**C**) No differences of plasma PAI-1 levels were observed in those with and without MACE excluding death (4918 pg/mL [2858-7187], n=76 vs. 5245 pg/mL [3413-7844], n=834; p=0.13). (**D**) No difference in plasma PAI-1 levels was observed in death (5988 pg/mL [3166-8068], n=37 vs. 5226 pg/mL [3365-7707], n=873; p=0.74). (**E**) No difference in plasma PAI-1 levels was observed in myocardial infarction (6427 pg/mL [3333-7411], n=19 vs. 5223 pg/mL [3364-7727], n=891; p=0.72). (**F**) No difference in plasma PAI-1 levels was observed in stroke (6281 pg/mL [2793-7710], n=17 vs. 5226 pg/mL [3373-7707], n=893; p=0.97). Data is presented as a violin plot and Mann-Whitney U-tests were conducted for the non-parametric data. P<0.05 is considered statistically significant. *P<0.05.

4.5.2 Baseline Plasma PAI-1 Levels and Clinical Events

During a median follow-up period of 12.4 months (IQR, 10.3 to 14.1 months), 103 patients (11.3%) experienced MACE, including 49 UR (5.4%) and 19 MI (2.1%). Patients who experienced unplanned revascularization had higher rates of diabetes, past history of percutaneous coronary intervention and myocardial infarction, and more likely to be on statins (Table 4-3).

Interestingly, among those with UR, 21 patients (42.9 %) who re-intervened due to in-stent restenosis had a median plasma PAI-1 level of 4989.4 pg/mL (IQR, 2538.4-6568.5 pg/mL). Furthermore, 25 patients (61.2%) who underwent a non-target lesion revascularization at the time of UR had a median PAI-1 level of 5257.9 pg/mL (IQR, 2980.7-6801.8 pg/mL) and 3 patients (6.1%) were revascularized for stent thrombosis (Figure 4-3).

Plasma PAI-1 level was not independently associated with UR nor MACE (Figure 4-4A and B). However, when plasma PAI-1 levels were separated into tertiles and adjusted for clinical and demographic variables (age, sex, diabetes, acute coronary syndrome, and time of procedure), PAI-1 levels were associated with UR after using the Cox proportional hazards (tertile 2: hazard ratio, 0.42; 95% CI: 0.15 to 1.18; tertile 3: hazard ratio 0.20; 95% CI: 0.05 to 0.86, Model 3, Table 4-4 and Figure 4-4A). Finally, tertiled plasma PAI-1

levels were not associated with individual components of MACE (

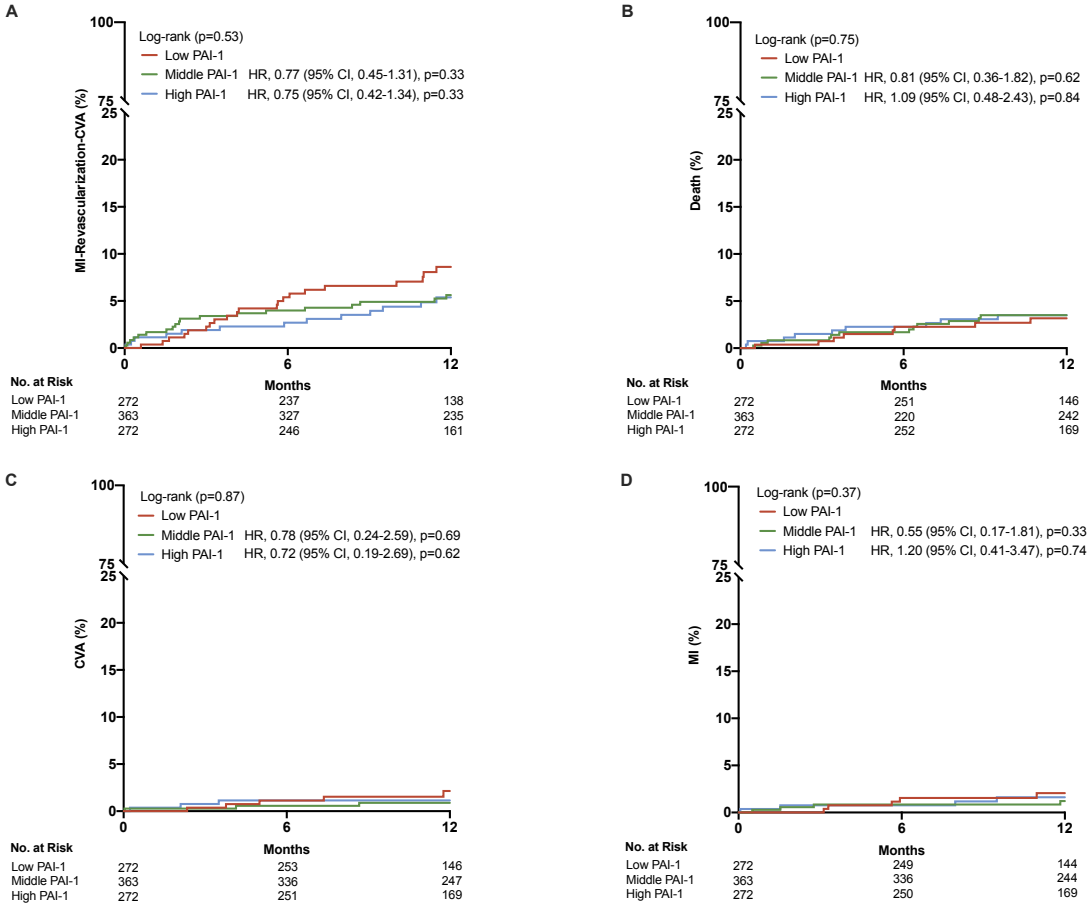


Figure 4-5).

The primary outcome of unplanned revascularization varied in the two periods between 0 to 30 days (tertile 2: hazard ratio, 4.49; 95% CI: 0.54 to 37.26; tertile 3: hazard ratio, 3.11; 95% CI: 0.32 to 29.93) and 30 days to 1 year (tertile 2: hazard ratio, 0.49; 95% CI: 0.24 to 0.99; tertile 3: hazard ratio, 0.36; 95% CI: 0.15 to 0.85) (Table 4-5). Despite differences in unplanned revascularization rates observed in the peri-procedural and 30 days to 1-year, other endpoints of interest were not associated with plasma PAI-1 the landmark analysis.

Table 4-3. Differences in baseline characteristic of those with unplanned revascularization

	Unplanned Revascularization (n=910)		
	No (n=861)	**Yes (n=49)**	**P-value**
Age (years) - mean +/- SD	66.4 ± 11.6	66.1 ± 11.9	0.86
Sex (female) - no. (%)	218 (25.3)	16 (32.7)	0.25
Body mass index (kg/m^2) - mean +/- SD	29.1 ± 5.7	28.7 ± 4.8	0.60
Hypertension - no. (%)	535 (62.1)	34 (69.4)	0.31
Dyslipidemia - no. (%)	526 (61.1)	34 (69.4)	0.25
Diabetes - no. (%)	210 (24.4)	22 (44.9)	0.0015
Smoking - no. (%)			0.65
Never	479 (55.6)	30 (61.2)	
Remote (quit >1 month ago)	237 (27.5)	13 (26.5)	
Active	145 (16.8)	6 (12.2)	
Family history of CAD - no. (%)	136 (15.8)	7 (14.3)	0.78
Atrial fibrillation - no. (%)	83 (9.6)	2 (4.1)	0.19
Indications for angiography - no. (%)			
ACS	359 (41.7)	24 (49.0)	0.32
Staged PCI	116 (13.5)	10 (20.4)	0.17
Stable CAD	321 (37.3)	15 (30.6)	0.35
Shock	1 (0.1)	0 (0.0)	0.81
Previous History - no. (%)			
PCI	232 (26.9)	27 (55.1)	<0.0001
MI	195 (22.6)	20 (40.8)	0.0036
CABG	47 (5.5)	4 (8.2)	0.42
PAD	57 (6.6)	1 (2.0)	0.20
CVA	44 (5.1)	5 (10.2)	0.12
Bleed	14 (1.6)	0 (0.0)	0.37
Heart failure	55 (6.4)	2 (4.1)	0.52
Medications - no. (%)			
ASA	791 (91.9)	48 (98.0)	0.12
P2Y12	792 (92.0)	48 (98.0)	0.13
ACEi/ARB	476 (55.3)	27 (55.1)	0.98
B-blocker	536 (62.3)	33 (67.3)	0.47
Calcium Channel Blocker	106 (12.3)	9 (18.4)	0.21
Statin	709 (82.3)	46 (93.9)	0.04
PPI	162 (18.8)	9 (18.4)	0.94
NSAID	10 (1.2)	0 (0.0)	0.45
Revascularized (stent + CABG) - no. (%)	546 (63.4)	34 (69.4)	0.40

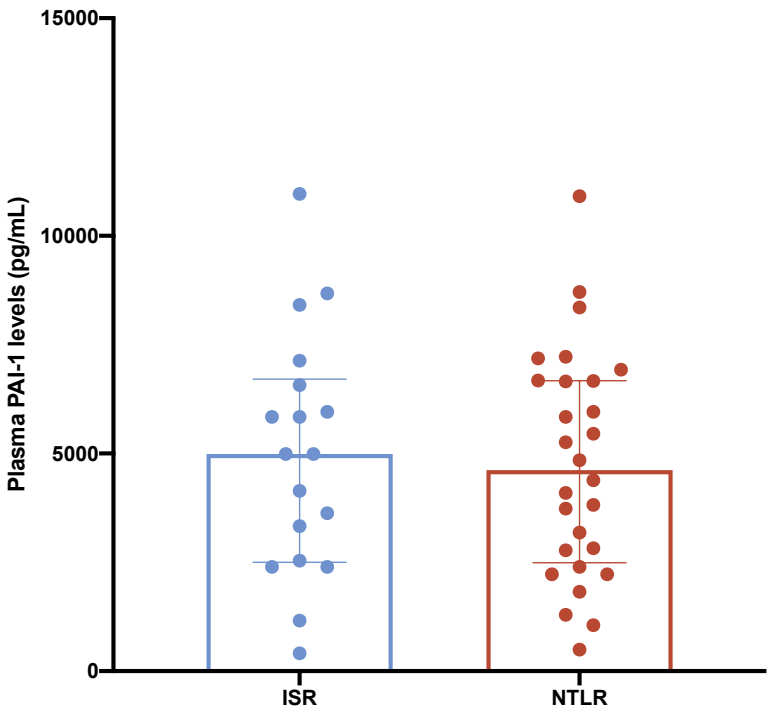


Figure 4-3. Baseline plasma plasminogen activator inhibitor-1 (PAI-1) levels stratified by type of unplanned revascularization.

Patients with unplanned revascularization due to in-stent restenosis (ISR) had a median PAI-1 level of 4989.4 pg/mL (IQR, 2538.4-6568.5 pg/mL). Finally, patients with non-target lesion revascularization (NTLR) had a median PAI-1 level of 5257.9 pg/mL (IQR, 2980.7-6801.8 pg/mL)

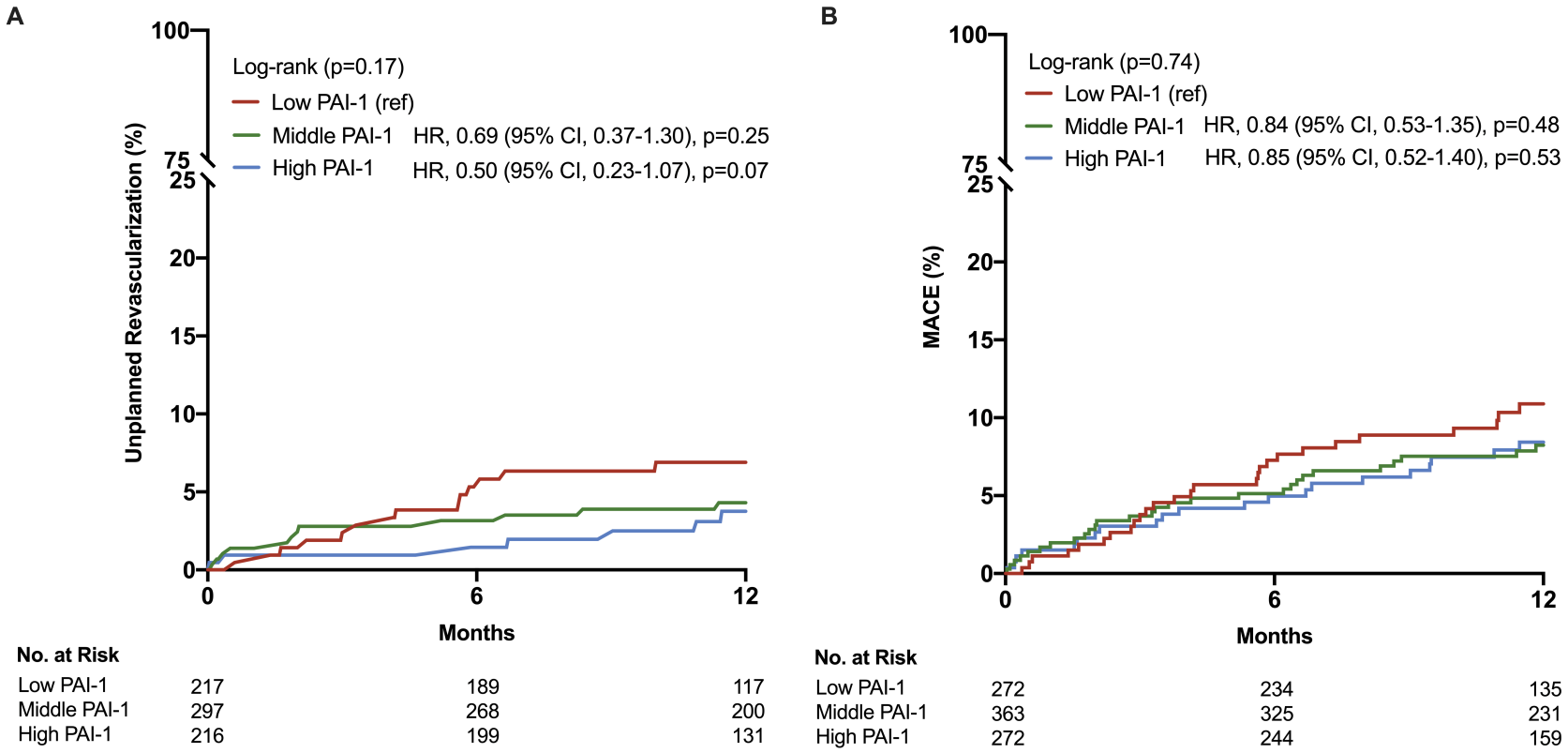


Figure 4-4. Kaplan-Meier curves of clinical outcomes after stratification of plasma PAI-1 levels into tertiles.

(**A**) Patients with cumulative incidence of unplanned revascularization within one-year follow-up separated by baseline PAI-1 measurements. Neither high nor middle tertile of plasma PAI-1 levels had lower incidence of unplanned revascularization. (**B**) Patients with cumulative incidence of major adverse cardiac events (MACE) within one-year follow-up separated by baseline PAI-1 measurements. Neither high nor middle tertile of plasma PAI-1 levels had lower incidence of MACE. Kaplan-Meier curves were generated and compared by log-rank test and hazard ratios were evaluated using the Cox proportional hazards model. $P<0.05$ is considered statistically significant

Table 4-4. Clinical outcomes predicted by plasma PAI-1 levels

	Unplanned revascularization		MACE	
Model	**HR (95% CI)**	**P-value**	**HR (95% CI)**	**P-value**
Model 1 - unadjusted tertile PAI-1 (relative to low PAI-1)				
Middle PAI-1	0.69 (0.37-1.30)	0.25	0.84 (0.53-1.35)	0.48
High PAI-1	0.50 (0.23-1.07)	0.07	0.85 (0.52-1.40)	0.53
Model 2 - adjusted tertile PAI-1 (relative to low PAI-1)*				
Middle PAI-1	0.66 (0.35-1.25)	0.20	0.78 (0.49-1.25)	0.30
High PAI-1	0.47 (0.21-1.03)	0.06	0.78 (0.47-1.30)	0.35
Model 3 - adjusted tertile PAI-1 (relative to low PAI-1)**				
Middle PAI-1	0.42 (0.15-1.18)	0.10	0.51 (0.25-1.04)	0.06
High PAI-1	0.20 (0.05-0.86)	0.03	0.47 (0.22-1.02)	0.057

*adjusted for age (years), acute coronary syndrome, type 2 diabetes, and sex

**adjusted for age (years), acute coronary syndrome, type 2 diabetes, sex, and time of procedure

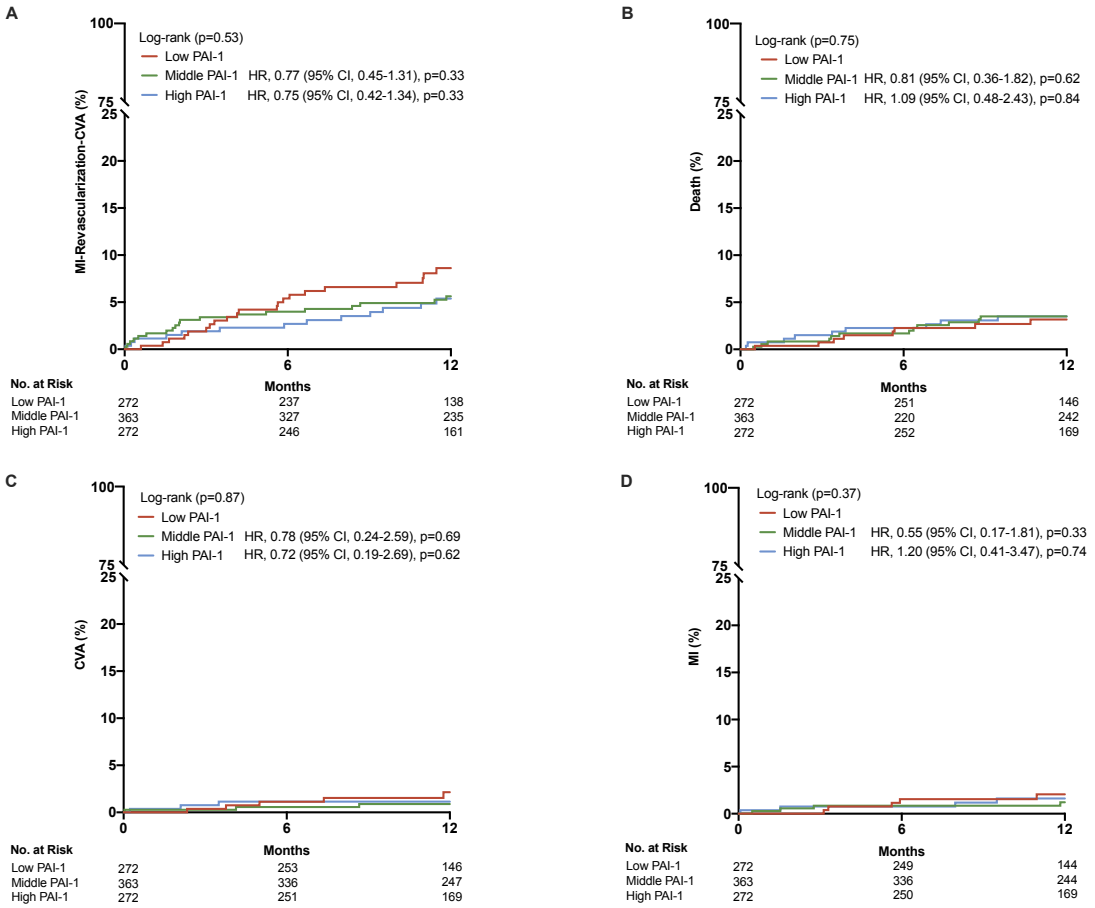


Figure 4-5. Cumulative incidence curves of individual components of major adverse cardiac events stratified by plasma PAI-1 levels.

(**A**) Cumulative incidence curve of MACE without death separated by tertiled PAI-1 levels. (**B**) Cumulative incidence curve of death separated by tertiled PAI-1 levels. (**C**) Cumulative incidence curve of cerebrovascular accident separated by tertiled PAI-1 levels. (**D**) Cumulative incidence curve of myocardial infarction separated by tertiled PAI-1 levels

Table 4-5. Primary and secondary outcomes in early- and late- stent complications

	Middle PAI-1 tertile			High PAI-1 tertile		
Variable	**Events (%)**	**HR (95% CI)**	**P-value**	**Events (%)**	**HR (95% CI)**	**P-value**
Early complications - <30 days						
Unplanned revascularization	*2.1*	4.49 (0.54-37.26)	0.16	1.4	3.11 (0.32-29.93)	0.33
MACE	*2.8*	2.56 (0.70-9.30)	0.15	2.3	2.06 (0.51-8.23)	0.31
Death	*0.8*	2.28 (0.24-21.90)	0.48	1.1	3.08 (0.32-29.57)	0.33
Myocardial infarction, unplanned revascularization, or stroke	*2.2*	6.13 (0.77-48.98)	0.09	1.9	5.14 (0.60-44.01)	0.14
Late complications - 30 days to 1 year						
Unplanned revascularization	*4.9*	0.49 (0.24-0.99)	0.048	3.4	0.36 (0.15-0.85)	0.02
MACE	*8.6*	0.68 (0.40-1.13)	0.14	9.2	0.73 (0.42-1.26)	0.25
Death	*2.8*	0.67 (0.28-1.62)	0.37	3.8	0.89 (0.37-2.15)	0.80
Myocardial infarction	*1.1*	0.43 (0.12-1.52)	0.19	2.3	0.87 (0.28-2.73)	0.81
Stroke	*1.4*	0.62 (0.18-2.18)	0.46	1.5	0.34 (0.07-1.79)	0.20
Myocardial infarction, unplanned revascularization, or stroke	*6.0*	0.55 (0.31-1.00)	0.05	6.2	0.57 (0.30-1.08)	0.09

*reference of low plasma PAI-1 tertile

4.5.3 Systematic Review of the Association Between Plasma PAI-1 and MACE after PCI

Study Selection

An updated literature search was conducted on December 18, 2019 and a total of 9612 articles were identified. After excluding 3178 duplicate articles, 6434 titles and abstracts were screened, of which 377 articles underwent full review. A total of ten articles were included for analysis (Figure 4-6).

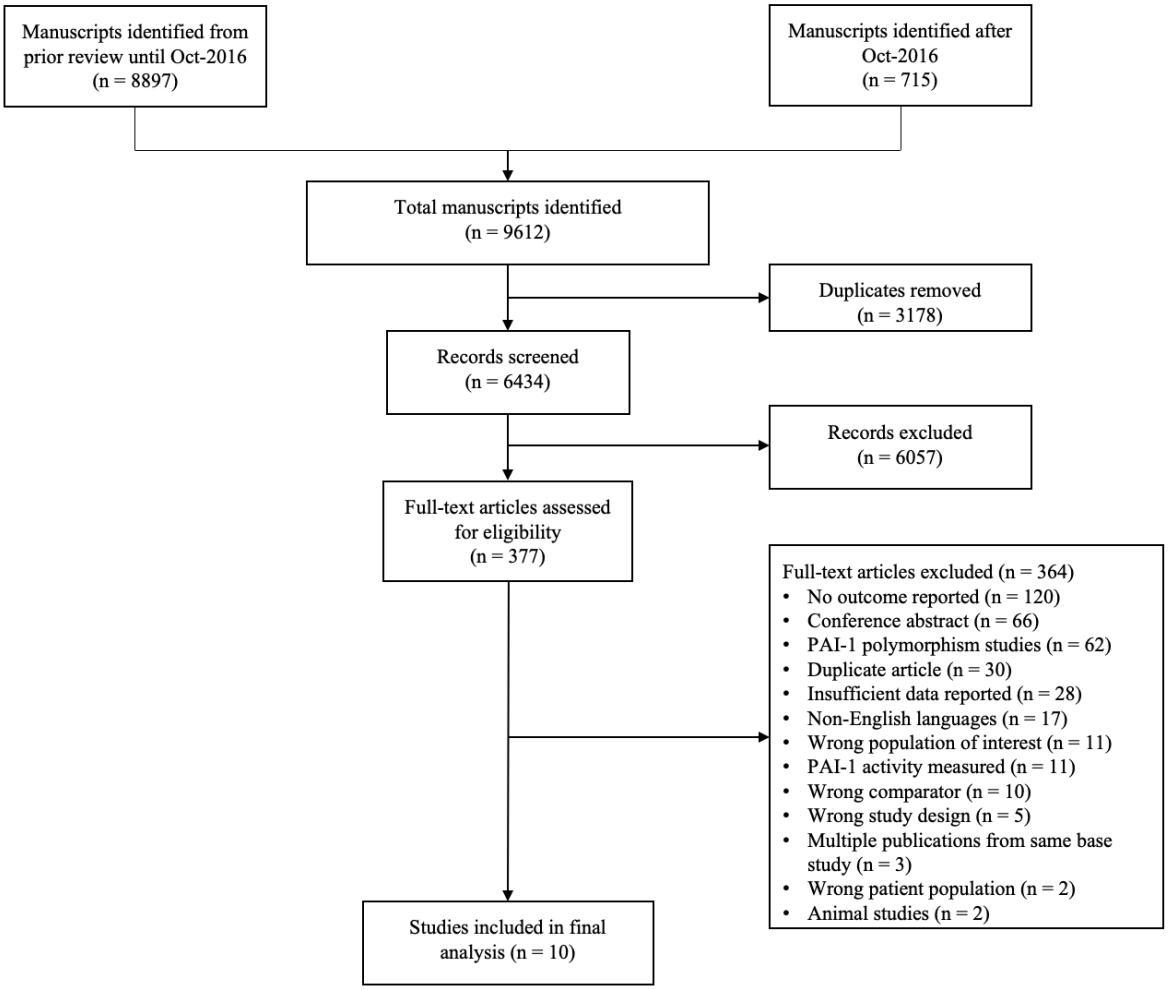


Figure 4-6. Flow diagram of selection of plasma PAI-1 studies for systematic review and meta-analysis

Plasma PAI-1 and Clinical Outcomes

Plasma PAI-1 levels were extracted from each manuscript with the corresponding event rates (Table 4-6**)**. Study sample sizes ranged from 42 to 2806 patients. Three trials examined the relationship between baseline plasma PAI-1 levels and MACE following PCI. Most studies were of moderate quality (Table 4-7). Considerable statistical heterogeneity was observed between studies (I^2 ≥79%). Pooled analyses failed to show an association between plasma PAI-1 and MACE in all-comer (mean difference: 2.29 ng/mL (95% CI, -0.81 to 5.38 ng/mL, P=0.15, Figure 4-7A)[147, 166, 210-217] or PCI-only populations (mean difference: 6.17 ng/mL (95% CI, -4.12 to 16.46 ng/mL, P=0.24, Figure 4-7B).[166, 213, 215]

Table 4-6. Included studies reporting plasma PAI-1 antigen levels (ng/mL) and major adverse cardiac events

				Event						No Event		
Reference	Year	Follow-up (months)	Population	N	PAI-1 (ng/mL)	Range	Death	MI	CVA	N	PAI-1 (ng/mL)	Range
Juhan-Vague et al.	1996	24	MI	106	18.2	8.2	40	66	n/a	2700	14.8	8.9
Moss et al.	1999	26	MI	81	25	18	25	56	n/a	964	29	28
Redondo et al.	2001	24	MI	37	40.725	22.28	2	35	n/a	157	42.65	21.02
Ganti et al.	2002	n/a	MI	4	80.68	16.38	4	n/a	n/a	38	61	21.95
Robinson et al.	2007	5 to 51	CAD	19	36.3	17.9	2	2	2	79	45.4	25.6
Pineda et al.	2010	36	MI	25	65.13	53.3	4	21	n/a	117	70.1	48.34
Yano et al.	2013	20	Smokers	66	65.73	64.57	n/a	11	55	744	42.97	36.54
Yano et al.	2014	30	HTN	42	31.67	16.12	4	13	16	548	28.33	14.87
Golukhova et al.	2015	28	Elective PCI	23	72.75	29.86	2	20	n/a	71	49.75	23.16
Kosaki et al.	2018	27	ACS	112	44.1	42.9	12	5	15	288	37.5	37.5

Table 4-7. Study quality of included studies examined using Newcastle-Ottawa Scale

Reference	Year	Study Design	Selection	Comparability	Exposure/Outcome	Total
Juhan-Vague et al.	1996	Cohort	3	0	3	6
Moss et al.	1999	Cohort	2	0	3	5
Redondo et al.	2001	Cohort	3	0	2	5
Ganti et al.	2002	Cohort	2	0	1	3
Robinson et al.	2007	Cohort	3	0	3	6
Pineda et al.	2010	Cohort	3	0	3	6
Yano et al.	2013	Cohort	3	0	3	6
Yano et al.	2014	Cohort	3	0	3	6
Golukhova et al.	2015	Cohort	2	0	3	5
Kosaki et al.	2018	Cohort	4	0	3	7

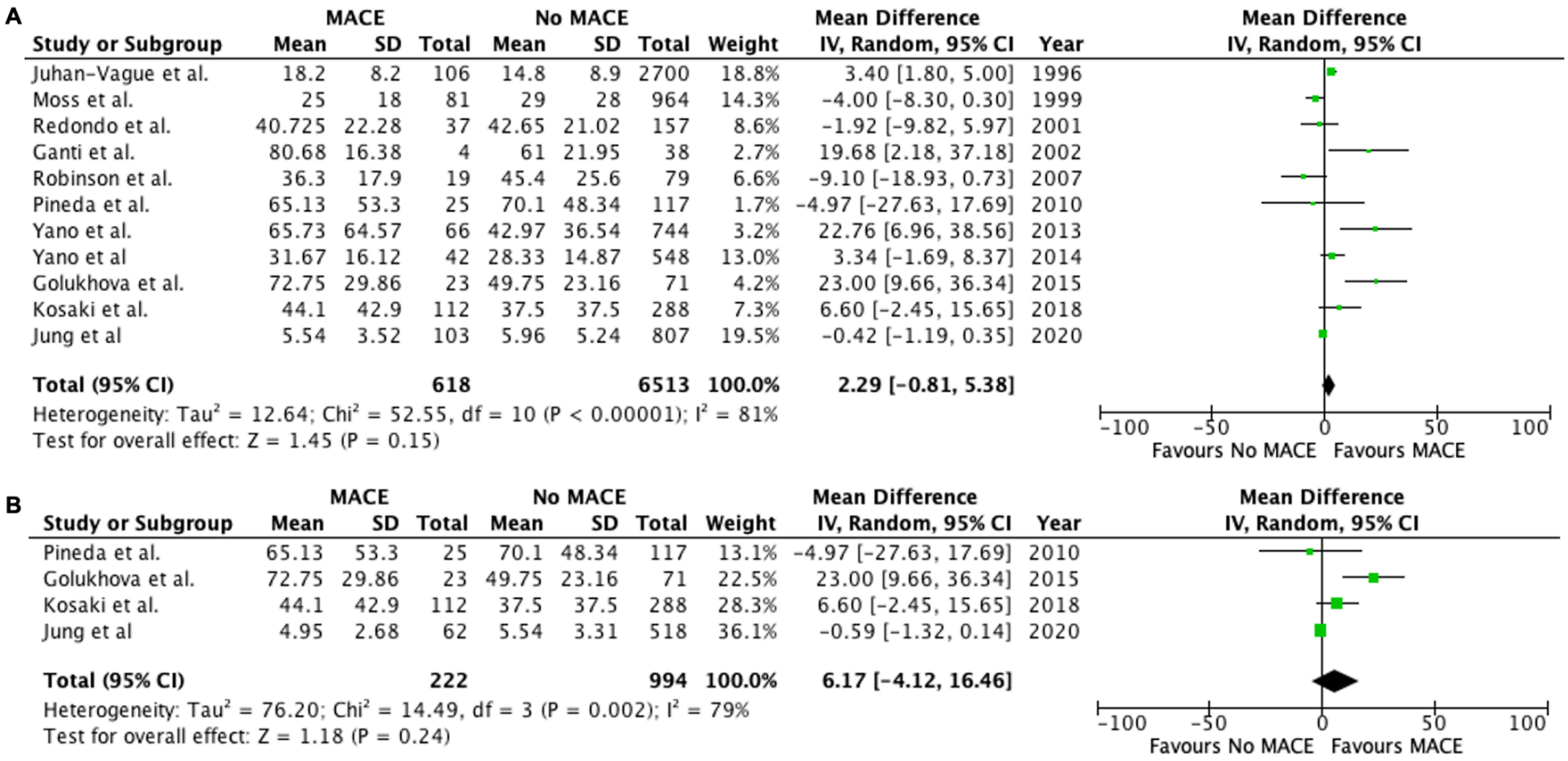


Figure 4-7. Comparison of mean plasma PAI-1 levels (pg/mL) with major adverse cardiac events (MACE).

(**A**) In all-comer population, no difference was observed between MACE and control with a mean difference of 2.29 ng/mL (95% CI, -0.81 to 5.38 ng/mL), p=0.15. (**B**) In percutaneous coronary intervention only population, no difference was observed between MACE and control with a mean difference of 6.17 ng/mL (95% CI, -4.12 to 16.46 ng/mL), p=0.24. Data is expressed as mean difference and analyzed using a random effects model. P<0.05 is considered statistically significant

4.6 Discussion

Our report is the largest cohort of patients undergoing coronary angiography and/or PCI with contemporary medical management. In this study, plasma PAI-1 was not found to be an independent predictor of UR nor of MACE but was associated with UR after adjustment for known clinical factors. Furthermore, in an updated systematic review and meta-analysis, plasma PAI-1 was not found to predict MACE in either the all-comer or PCI-only population.[205] Finally, *post-hoc* landmark analysis suggested that low PAI-1 levels were associated with increased risk of stent complications beyond the peri-procedural period.

In our angiography-based study, the lack of association between plasma PAI-1 and MACE stands in contrast to previously published work.[25, 202, 210] Importantly, previous studies had a long-term follow-up period exceeding 10 years compared to the one year follow up in our current report. Furthermore, these studies were conducted in populations without pre-existing cardiovascular disease whereas our cohort consists of patients with a high pre-test probability of CAD presenting for revascularization. Interestingly, our landmark analyses suggested that PAI-1 was not predictive in the acute post-procedural period but was predictive of late complications in the period where ISR remains predominant. If confirmed, this finding may be owing to differences in the biology of early and late vessel failure. Importantly, the lack of association between MACE and PAI-1 observed in our study is not likely due to the existence of known risk factors which influence plasma PAI-1 levels such as age, type 2 diabetes, obesity, and acute coronary syndrome, as adjusted analysis failed to identify significant associations.[48, 96, 218] Finally, it is important to note that we did not measure PAI-1 activity or its target tPA as measurement of activity levels are sensitive to experimental factors and technical challenges such as isolation technique used, temperature, and time of blood draw. [39, 85, 219] Furthermore, PAI-1 antigen measurement captures

all variants of PAI-1 including: 1) free and active PAI-1 (measured in a PAI-1 activity assay), 2) PAI-1 complexed to tPA or uPA (measured in both PAI-1 and tPA antigen ELISA), and 3) latent or inactive PAI-1.[205] Nonetheless, the totality of evidence currently does not support plasma PAI-1 as predictive of MACE in either all-comer or PCI-only populations.

Previous studies have demonstrated that low levels of plasma PAI-1 were associated with higher rates of ISR following PCI.[180, 220-222] Our results complement these studies with reduced plasma PAI-1 levels are associated with unplanned revascularization. Moreover, landmark analysis for late complications suggested that low plasma PAI-1 levels were associated with late unplanned revascularization. Follow-up beyond the peri-procedural period is known to be a high-risk period for in-stent restenosis with peak neointimal formation occurring 6 months following PCI.[209, 223] This result is not surprising given our previous meta-analysis which revealed that increased rates of coronary restenosis following PCI was associated with reduced levels of plasma PAI-1.[180, 205, 221, 224]

In our cohort, plasma PAI-1 levels were associated with unplanned revascularization following adjustment with clinical variables. Furthermore, *post-hoc* landmark analysis conducted beyond one-month following angiography demonstrates that high plasma PAI-1 levels were associated with reduced rates of unplanned revascularization. Contemporary molecular understanding of in-stent restenosis is believed to be driven by the recruitment of inflammatory cells beyond the peri-procedural period of one-month, driving vascular SMC phenotypic switching to a hyper-proliferative and migratory state to narrow and eventually obstruct the vascular lumen to cause ISR.[108] Interestingly, elevated levels of PAI-1 has previously been associated with decreased ISR rates in patients following PCI.[180, 205, 220] Moreover, anti-proliferative agents such as paclitaxel coated onto stents induced PAI-1 expression to prevent restenosis by complexing

with vitronectin to inhibit the pro-inflammatory environment required for SMC phenotypic switch.[203, 225, 226] Patients in our cohort with unplanned revascularization experienced stent failure and the measurement of plasma PAI-1 at the time of catheterization potentially has the additive effect of identifying high-risk populations following PCI since no established risk scores yet exists. These findings remain hypothesis generating and future studies examining the relationship of plasma PAI-1 following PCI should focus on the predictive ability of PAI-1 and ISR beyond one-month.

The Kaplan-Meier curve examining the relationship between plasma PAI-1 and unplanned revascularization in the peri-procedural period favour low levels of plasma PAI-1. Following plaque erosion or rupture – as it potentially may occur during stent deployment - platelets are recruited to the site of injury and release PAI-1 into the vascular milieu creating a pro-thrombotic environment.[68] Pathologically elevated levels of PAI-1 has been associated with lysis-resistant thrombus.[227] Moreover, since PAI-1 levels are correlated with platelet activation, it may behave as a surrogate marker of pro-thrombotic extracellular vesicle release at the site of vascular injury.[228] Given that PAI-1 as a biomarker appears to behave in a paradoxical fashion in the short- vs long-term post-PCI, the question pertaining to the utility of plasma PAI-1 as a biomarker in predicting UR and MACE requires refinement of when it is measured and of the population of interest. Importantly, it does not exclude a potential to target PAI-1 for therapeutic efficacy, especially in high risk periods.

The poor predictive ability of plasma PAI-1 with MACE may be due to multiple factors, including: (1) circulating PAI-1 levels are influenced by circadian rhythm,[85] (2) its levels are affected by various pro-inflammatory states which are predominant in our cohort,[206] (3) no reference range or specific cut-off values for plasma PAI-1 levels has yet been established, and (4)

PAI-1 levels may not be predictive of MACE in those with established CAD. We have previously demonstrated that since PAI-1 had a low index of individuality of 0.4, changes in serial measurements of plasma PAI-1 levels from multiple samples are more meaningful to ascertain risk.[206] Accordingly, studies of PAI-1 as a biomarker focused on serial changes rather than absolute values may be informative based on our current findings.

Our study is not without limitations. First, the influence of circadian rhythm on plasma PAI-1 levels is not accurately captured by accounting for daytime variation in coronary angiography performed due to variance in diurnal rhythm.[85] Second, although a prospective registry data collected at the time of catheterization was used to capture clinical outcomes, this remains a observational study and the plasma levels are potentially influenced by other confounding variables, which may not have been captured in the analysis. Third, our study measured only baseline plasma PAI-1 levels at the time of angiography and did not measure serial changes throughout the follow-up period. Fourth, the association between plasma PAI-1 levels and unplanned revascularization remains exploratory and requires validation.

4.7 Conclusion

Basal plasma PAI-1 levels are not independently associated with UR but not MACE in patients undergoing angiography and/or PCI but associated with UR following adjustment with known clinical factors. Moreover, our *post-hoc* landmark analysis revealed that low plasma PAI-1 levels were associated with increased UR rates for late complications. Our findings suggest that studies focusing on serial change from multiple measurements rather than basal plasma PAI-1 levels may be informative and shed light on the mediatory role of PAI-1 in the pathogenesis of stent complications following PCI.

4.8 Sources of Funding

This project was supported by the Innovation Fund of the Alternative Funding Plan for the Academic Health Sciences Centres of Ontario and Canadian Foundation of Innovation. R.G.J. was funded by the Vanier Canadian Institutes of Health Research (CIHR) Canada Graduate Scholarship for his graduate studies. A.C.D. was supposed by the Cardiac Endowment Fund at the University of Ottawa Heart Institute. F.D.R. was supported by a CIHR Banting Postdoctoral Fellowship and a Royal College of Physicians and Surgeons of Canada Detweiler Travelling Fellowship.

4.9 Conflict of Interest

On behalf of all authors, the corresponding author states there are no conflicts of interest for the completion of the manuscript

Chapter 5

Plasminogen activator inhibitor-1 platelet extracellular vesicles predicts MACE and the pro-inflammatory SMC phenotype

5.1 Preface

This chapter has been submitted and is currently under review.

5.2 Abstract

Background: Patients with established coronary artery disease (CAD) remain at elevated risk of major adverse cardiac events (MACE). We sought to evaluate the utility of plasminogen activator inhibitor-1 positive platelet-derived extracellular vesicles (PAI-1^+ PEV) as a biomarker for MACE, and to explore potential underlying mechanisms.

Methods: Circulating plasma PAI-1^+ PEV levels were assessed by flow cytometry and electron microscopy in 456 patients with CAD. The utility of PAI-1^+ PEV as a biomarker for MACE at one year was evaluated in a discovery and validation cohort. Mechanisms of PAI-1^+ PEV-induced changes to vascular smooth muscle cells (VSMC) and thrombus formation was assessed *in vitro*.

Results: PAI-1^+ PEV levels were elevated in MACE (13786.6 [6711.7-26942.1] vs. 8344.9 [3777.2-33499.7] PAI-1^+ PEV/μL; p=0.047; for event vs. non-event, respectively). Patients with high circulating levels of PAI-1^+ PEV had higher rates of MACE (262.3 vs. 103.0 events per 1,000 person-years; hazard ratio (HR) 2.19; 95% CI, 1.07-4.52; and HR 2.67; 95% CI, 1.22-5.84, discovery and validation cohorts, respectively). High PAI-1^+ PEV fractions did not enhance thrombogenicity but promoted a pro-inflammatory VSMC state by enhancing proliferation and

migration, and up-regulating inflammatory genes (KLF4). Inhibition of the PAI-1-LRP-1 interaction dampened the pro-inflammatory VSMC response.

Conclusion: Circulating PAI-1$^+$ PEV induced pro-inflammatory changes in VSMC through LRP1 and is strongly associated with MACE. Targeting the PAI-1$^+$ PEV-VSMC interaction may offer a novel target to modulate cardiac events in patients with CAD.

5.3 Introduction

Obstructive coronary artery disease (**CAD**) is frequently treated with revascularization by percutaneous coronary intervention (**PCI**) or coronary artery bypass grafting (**CABG**) in patients with refractory symptoms or acute coronary syndromes.[1, 229]. Despite significant advances in PCI techniques and technologies since its inception, the stented coronary artery remains the highest-risk coronary lesion with annualized adverse event rates as high as 8-12% in the following year.[114, 118, 199] Comparatively, the putative vulnerable plaque has an annualized event rate of 0.05% - making identification and treatment of patients following coronary stenting an unmet need.[114, 118, 199] Two major complications resulting in target vessel failure are in-stent restenosis (**ISR**) and stent thrombosis (**ST**).[223]

Plasminogen activator inhibitor-1 (**PAI-1**) is primarily an anti-fibrinolytic protein which inhibits fibrin clot degradation by preventing the action of tissue-type plasminogen activator.[203] Elevated levels of PAI-1 has been previously associated with type 2 diabetes, acute myocardial infarction (**MI**) and unplanned revascularization.[48, 205, 230] Although PAI-1 is synthesized and secreted by endothelial cells, cardiomyocytes, and fibroblasts, the majority of plasma PAI-1 is derived from platelets.[48, 68, 203] Interestingly, inhibition of PAI-1 has previously been demonstrated

to reduce vascular smooth muscle cell (**VSMC**) migration and resultantly mitigate intimal formation after vascular injury in mouse models.[74, 231] However, in humans few studies have evaluate or linked PAI-1 levels to outcomes in patients with CAD.

Extracellular vesicles (**EVs**) are released from circulating blood cells and vascular cells at the time of vascular injury.[228, 232, 233] In addition to small studies suggesting a role as a biomarker, EVs potentially modulates stent failure in two ways: 1) EVs are abundant in surface-exposed negatively-charged phosphatidylserine which catalyzes thrombosis,[234] and 2) EVs contain pro-inflammatory protein and genetic material that could be potentially transferred to recipient cells at sites of vascular injury to modulate a phenotypic response.[235-239] To date, no data exists on the relationship between PAI-1 and EVs in humans despite platelets being a common source – or if they play a pathological role in clinical events in patients with CAD.

Accordingly, we hypothesized that platelet-derived extracellular vesicles may represent a major source of pathologic PAI-1 (**PAI-1$^+$ PEV**). Moreover, we evaluated if these EVs were associated with major adverse cardiac events (**MACE**). Finally, sought to evaluate the role of PAI-1+ PEVs in models of thrombus formation and in VSMC phenotypic switching for arterial remodeling post revascularization.

5.4 Methods

5.4.1 Ethics statement

This study was approved by the Ottawa Health Science Network Research Ethics Board (#20160516-01H for *in vitro* experimentation and #20190224-01H for clinical follow-up). Written informed consent was obtained from all participants.

5.4.2 Study population and sample collection

The University of Ottawa Heart Institute is a regional tertiary center serving over 1.2 million people in the capital region of Canada and all coronary catheterization procedures are registered in the Cardiovascular And Percutaneous clInical TriALs (CAPITAL) revascularization registry. This registry captures over 1,200 clinical data points with regards to patient and procedural factors.[207] CAD was defined as angiography confirmed stenosis ≥50%. Diabetes status was defined as having hemoglobin A1c levels ≥6.5% on presentation or prior diagnosis of diabetes mellitus (**DM**).[206] Patients undergoing revascularization after hours were not included for workflow reasons.

Between October 2016 to August 2018, blood samples were collected on consecutive eligible patients referred for coronary revascularization.[230] Arterial blood samples were collected in 3.2% sodium citrate tubes (Becton Dickinson, Franklin Lakes, NJ, USA) immediately upon the completion of the procedure and processed within two hours. Samples were centrifuged at 1,200 x g for 10 minutes at 4°C without brakes from which plasma was extracted and spun at 3,200 x g for 10 minutes to remove further contaminants and was stored at -80°C until further processing.

5.4.3 Clinical outcomes

Outpatient clinic or chart review was conducted at one-year following basal blood draw to evaluate the endpoints of major adverse cardiac events and unplanned revascularization. The primary outcome was MACE, defined as a composite of death, myocardial infarction, unplanned revascularization, and cerebrovascular accident. Secondary outcomes were individual components of MACE. Clinical follow-up was conducted in-person and recorded in the CAPITAL Revascularization Registry. Clinicians who performed the clinical follow-up were blinded to PAI-

1^+ PEV levels. For analysis purposes, the entire cohort of consecutive 456 patients with PAI-1^+ PEV levels were equally divided into two groups by simple random sampling of events (n=76) and non-events (n=380) into a discovery and validation cohort (n=228 each).

5.4.4 Washed platelet preparation

Whole blood was spun at 200 x g for 20 minutes with no brakes and the top layer was carefully transferred into a falcon tube, PGE_1 was added to a final concentration of 1μM and was spun at 1,000 x g for 10 minutes with no brakes. Platelets were washed in washing buffer (103 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, 36 mM citric acid, 3.5 mg/mL BSA, and pH 6.5) and spun at 1,000 x g for 10 minutes. Finally, platelets were resuspended in Tyrode's buffer (5 mM HEPES, 137 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 3.5 mg/mL BSA, and pH 7.4).

5.4.5 Plasma PAI-1 assessment

Human plasma spun at 3,200 or 20,000 x g at 4°C were measured in a 1:5 dilution using human PAI-1 enzyme-linked immunosorbent assay was purchased from Abcam (ab184863, Abcam, Cambridge, United Kingdom) as per manufacturer's recommendations by a researcher blinded to patient and procedural characteristics. PAI-1 activity (IU/mL) was evaluated as per manufacturer's recommendations using a chromogenic assay kit (ab108894, Abcam).

5.4.6 Extracellular vesicle analysis

For isolation of EVs, plasma was first spun at 20,000 x g for 20 minutes. Then the sample was resuspended in Annexin V buffer and incubated with Annexin V-PE (130-118-363, Miltenyi Biotec), spun at 20,000 g for 20 minutes, and incubated with appropriate antibodies (1:20 CD41a-VioBlue (130-105-558) and 1:7.5 PAI-1-FITC (11501-05041) or IgG-FITC for 1 hour. Samples were spun at 20,000 g for 20 minutes and resuspended in phosphate-buffered saline (**PBS**), with 1:300,000 1.00 μm Fluoresbrite YG Microspheres (17154-10, Polysciences Inc). Data on 10,000 YG microsphere events were acquired using the MACSQuant 10 flow cytometer (Miltenyi Biotec). PAI-1$^+$ PEV (defined as Annexin V$^+$/CD41$^+$/PAI-1$^+$) quantification was conducted using FlowJo v10 (Ashland, Oregon, USA).

5.4.7 Electron microscopy

PEVs ($1.0x10^7$ EVs/mL) were incubated for 4 hours in PBS containing 0.05% bovine serum albumin and anti-PAI-1 monoclonal antibody (1:200). Following primary antibody incubation, the sample was washed with PBS and incubated for 1 hour in goat anti-mouse IgG gold-linked polyclonal antibody (1:50, ab27421, Abcam). The sample was washed in PBS and loaded onto carbon-coated copper grids (300 mesh) for transmission electron microscopy (Tecnai^{TM} G^2 Spirit TWIN, FEI Company, Hillsboro, USA).

5.4.8 Thrombus formation using the Total Thrombus Analysis System

Thrombus formation was analyzed using a validated automatic microchip-based flow chamber system Total Thrombus Analysis System (**T-TAS**, Zacros, Fujimori Kogyo Company, Japan), using the AR chip.[240-243] Whole blood was incubated with increasing concentrations of EVs isolated from volunteers for 5 minutes prior to T-TAS analysis. For comparative analysis

between ISR and control EV, plasma from ISR and control patients were spun at 20,000g for 90 minutes and a final concentration of $3.5x10^4$ EVs/uL was added to volunteer whole blood prior to analysis on T-TAS. Differences in thrombus formation was quantified at four points: 1) time to thrombus onset measured at 10 kPa (**T_{10}**), 2) time to occlusive thrombus measured at 80 kPa (**T_{80}**), 3) the rate of thrombus formation measured as a difference of T_{80} and T_{10}, and 4) total thrombogenicity quantified as area under the pressure vs. time curve.

5.4.9 Human coronary artery smooth muscle cell assessment

Human coronary artery smooth muscle cells (C0175C, ThermoScientific) were grown in Medium 231 (ThermoScientific) in growth supplement (S00725). $1.0x10^8$ washed platelets/mL isolated from healthy volunteers were stimulated overnight with 1.0 μg/mL collagen at room temperature in order to generate PEVs and quantified via flow cytometry. VSMCs were differentiated in differentiation supplement (S0085, ThermoScientific) for 72 hours in 6-wells and incubated with extracellular vesicles for 24 hours prior to extraction.

5.4.10 Real-time gene expression analysis

VSMCs were plated onto 6-well plates in Medium 231 and were subsequently treated with increasing concentrations of EVs ($1.5\text{-}6.0x10^4$ EVs/μL) and total RNA was extracted using TRIzol LS (10296010, ThermoScientific) at the indicated timepoints according to manufacturer's recommendations. Total RNA (750 ng) was used for reverse transcription using SuperScript™ IV VILO Master Mix (11766050, ThermoScientific) according the manufacturers' guidelines. cDNA (1:10 dilution) was used for real-time expression analysis via the Eppendorf RealPlex2 and

quantified by the Pfaffl method.[244] Primers for *KLF4, KLF5*, smooth muscle cell actin (*ACTA2*), and *GAPDH* were previously described.[245]

5.4.11 Cell proliferation and apoptosis

VSMC proliferation was measured using the CellTrace Violet kit (C34557, ThermoScientific). VSMCs were stained in the presence of 5 μM CellTrace Violet were seeded onto 96-well plates ($5.0x10^3$ VSMCs/mL) for 24 hours. They were then washed with PBS and incubated with increasing concentrations of PEVs ($1.5\text{-}10.0x10^4$ EVs/μL) and/or tiplaxtinin (SigmaAldrich) for 24 and 48 hours at 37°C and 5.0% CO_2. Proliferation and apoptosis (Annexin V-FITC kit, 130-092-052, Miltenyi Biotec) was quantified according to manufacturer's recommendations using the MACSQuant Analyzer 10.

5.4.12 Platelet EV interaction with human coronary VSMCs

Platelets ($1.0x10^8$/mL) were labelled with 10μM CellTracker Green CMFDA Dye (C2925, ThermoScientific) in Tyrode's Buffer for 20 minutes and spun at 1000 x g for 10 minutes without brakes. $CMFDA^+$ platelets were resuspended in Tyrode's Buffer and stimulated with 1.0 μg/mL collagen overnight to generated $CMFDA^+$ platelet EVs and quantified by flow cytometry. Subsequently, $1x10^8$ platelet EVs/mL were incubated with tiplaxtinin or TM5275 for 30 minutes at room temperature. Subsequently, they were co-incubated with human coronary VSMCs for 40 minute at room temperature as previously described by another group.[237] Free platelet EVs were removed following centrifugation at 1400 rpm and washed with PBS. Control incubation was done by co-incubating PEVs with VSMCs in both vehicle control or in the presence of EDTA. $CMFDA^+$ EV interaction with VSMC was quantified by median VSMC FITC intensity by flow cytometry.

5.4.13 Internalization of platelet EVs into VSMCs

Washed platelets ($1.0x10^8$ /mL) extracted from healthy volunteers was stained with CFSE for 20 minutes in Tyrode's buffer and spun at 3,200 x g for 20 minutes. Labelled platelets were subsequently stimulated with 1.0 μg/mL collagen overnight and platelets EVs were quantified using Annexin V and CD41. CellMask Orange labelled VSMCs plated on glass slips were co-incubated with CellTracker Green$^+$ EVs for 8, 12, and 24 hours and fixed with 4% paraformaldehyde and labelled with Hoescht staining dye. Three-dimensional images were acquired using the Zeiss Elyra LSM880 and analyzed using the Zen lite 3.1 and ImageJ.

5.4.14 Scratch assay

VSMCs were plated onto 96-well plates ($2x10^4$ cells/mL) in Medium 231 and incubated at 37°C and 5.0% CO_2 until confluence was achieved. VSMCs were then labelled with CellMask Orange (C10045, ThermoScientific) and washed with PBS. Cells were scratched using a p200 tip in a vertical direction, washed with PBS, and treatments suspended in Medium 231 were added to the 96-well plates and imaged every 2 hours using the Cytation 5 (BioTek, Winooski, Vermont, USA). Wound closure percentage was quantified on the Cytation Gen5 software using manufacturer recommendations.

5.4.15 Statistical analysis

Continuous variables were reported as mean ± standard deviation or median ± interquartile range (**IQR)** as appropriate. Continuous variables were compared by student t-test's

and multiple comparisons compared to DMSO or EV controls were evaluated by Dunnett's test. Categorical variables were reported as proportions (%). PEV and PAI-1$^+$ PEV levels were compared with standard t-tests following log transformation. Categorical variables were compared by Chi-Squares test or Fisher's exact test.

All patients included in the study completed their one-year follow-up. Receiver-operating characteristics (**ROC**) curves were generated and the Youden Index was identified to ascertain the optimal cut-off value of PAI-1$^+$ PEV in the discovery cohort and validated in the validation cohort.[246]

Kaplan-Meier curves were generated to evaluate time-to-event distributions and compared by log-rank tests. Hazard ratios (**HR**) and corresponding 95% confidence intervals were obtained from stratified Cox proportional-hazard models following adjustment by clinical characteristics (age, DM, and acute coronary syndrome) determined *a priori* based on clinical judgment.

All flow cytometry data was analyzed using FlowJo v10 (Becton, Dickinson & Company, Franklin Lakes, New Jersey, USA). All statistical analysis was performed using SAS v9.4 (SAS Institute, Inc., Cary, North Carolina, USA). ROC curves and reclassification were performed using the %ROCPLUS macro. All figures were created using GraphPad Prism v8 (GraphPad Software, La Jolla, California, USA). P-value less than 0.05 was considered statistically significant.

5.5 Results

5.5.1 PAI-1$^+$ PEVs in human circulation

Following isolation of extracellular vesicles from human plasma in CAD patients spun at 20,000 g, PAI-1$^+$ PEV (defined as Annexin V$^+$/CD41$^+$/PAI-1$^+$ large extracellular vesicles) were measured by flow cytometry after generating a size gate using standardized Apogee beads (#1493).

Gates for Annexin V, CD41, and PAI-1 were based on the use of isotype and fluorescence minus one control where appropriate (Figure 5-1). Localization of PAI-1 on the surface of PEV was further validated by electron microscopy and labeling with PAI-1 antibody and appropriate gold-linked IgG secondary antibody (Figure 5-1J). In order to determine the proportion of PAI-1 associated with EVs in human plasma, plasma was EV-depleted (Figure 5-1K, demonstrating the relative decrease in PAI-1 levels) and PAI-1 measured at baseline and post depletion showing that PAI-1^{+} PEV contributes to $19.1 \pm 6.3\%$ of PAI-1 levels found in plasma.

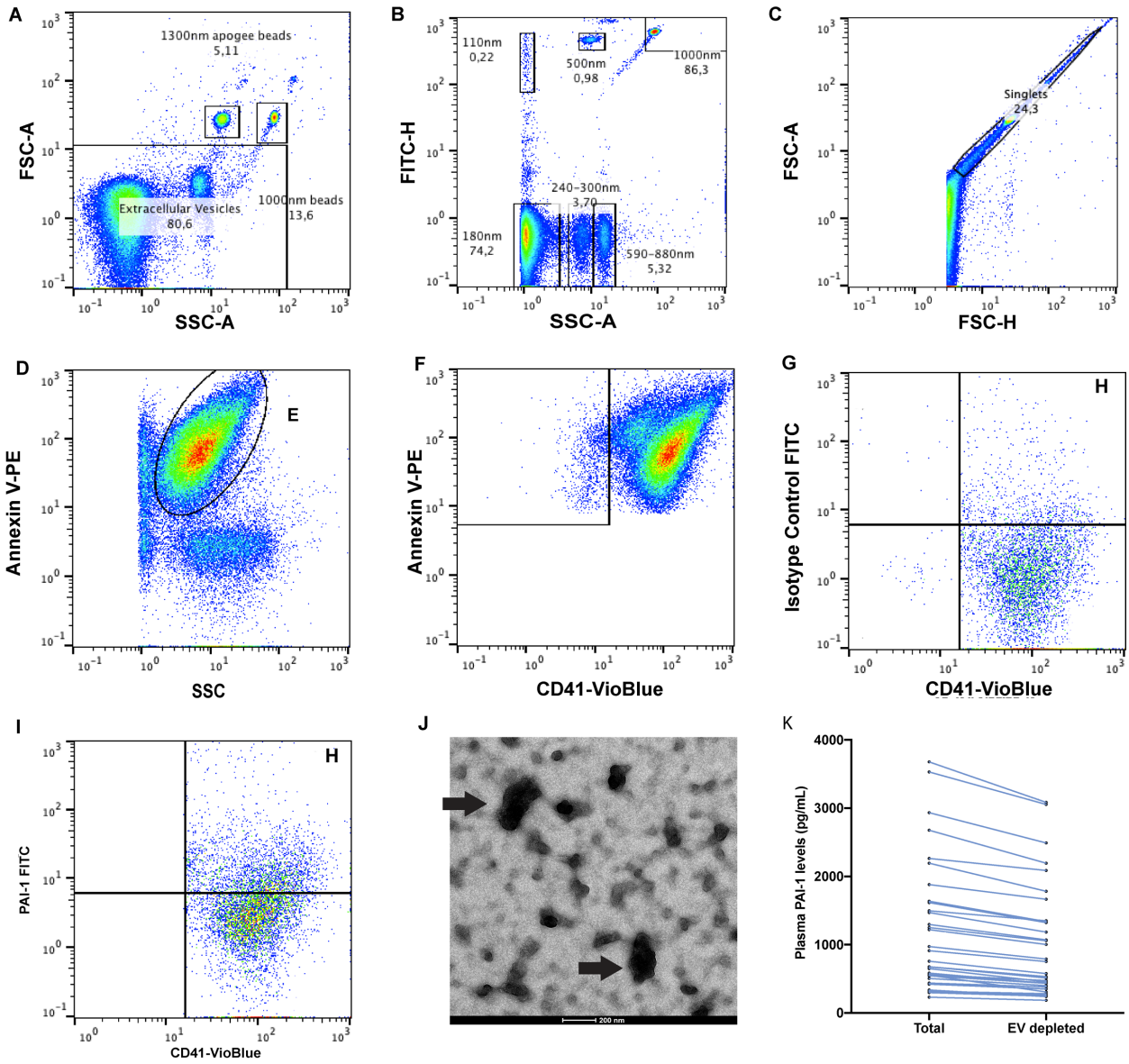


Figure 5-1. Flow cytometry gating profile to identify PAI-1$^+$ PEV.

(A/B) Forward and side-scatter gate to identify upper limit using Apogee Beads. Representative Fluoresbrite YG beads is labelled 1000 nm beads. (C) Singlet discrimination using FSC-A and FSC-H. (D) Annexin V-PE positive gate (gate labelled E) to isolate extracellular vesicles. (F) From Annexin V$^+$ gate, platelet extracellular vesicles were identified. (G) Gating profile of PAI-1$^+$ PEV identified **H-gate** using isotype control FITC. (J) PAI-1$^+$ PEV complex captured by electron microscopy following immunogold staining of PAI-1. (K) Plasma PAI-1 levels measured at baseline and EV-depleted samples. All gates were drawn based on fluorescence minus one control unless otherwise specified.

5.5.2 Plasma PAI-1$^+$ PEV does not impact thrombus formation

We hypothesized that PAI-1$^+$ PEV would mediate thrombosis as both PAI-1 and EVs independently propagate thrombus formation. Using the available clinical data, no relationship was observed between PAI-1$^+$ PEV levels in CAD patients with or without antiplatelet therapy nor was there an association between antiplatelets and MACE (Figure 5-2). Next, we evaluated the effect of PEVs on thrombogenicity using T-TAS.[241] Increasing concentrations of PEVs (1.5-6.0x10^4 PEV/μL) were added to healthy volunteer blood and was associated with rapid thrombus initiation and vessel occlusion along with increased thrombogenicity (Figure 5-3A-D). In order to determine the effect of PAI-1$^+$ PEV on thrombus formation, we isolated plasma EVs from high vs. low proportion of PAI-1$^+$ PEV patients and incubated healthy volunteer blood with a standardized amount of PEVs (3.5x10^4 PEVs/uL). The incubated blood was processed on the T-TAS where we observed no differences in time to onset of thrombus formation between high vs. low proportion of PAI-1$^+$ PEV (6.0 $\pm$ 0.3 min vs. 6.0 $\pm$ 0.7 min; p=0.80; Figure 5-3E) and time to occlusion (7.9 $\pm$ 0.2 min vs. 8.2 $\pm$ 1.5 min; p=0.63; Figure 5-3F). Moreover, the rate of thrombus formation, calculated as a difference from time of onset to occlusion, was not different between the two groups (1.9 $\pm$ 0.3 min vs. 1.8 $\pm$ 0.4 min; p=0.80; Figure 5-3G). Overall thrombogenicity was also similar between the two groups (1854.0 $\pm$ 18.0 AUC vs. 1798.0 $\pm$ 151.8 AUC; p=0.27; Figure 5-3H). Corresponding microscopic images of thrombus formation under AR-chip flow conditions are available in Figure 5-3I. Although increasing concentrations of PEV impacted thrombus formation, different proportions of PAI-1$^+$ PEV does not appear to impact thrombogenicity.

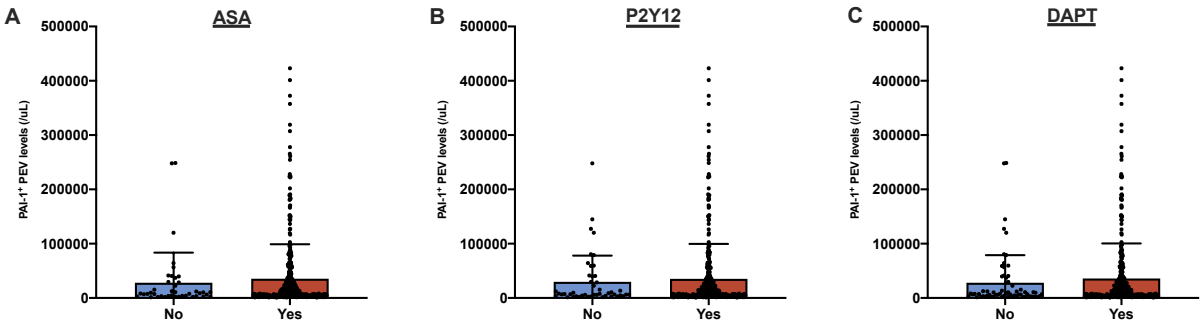


Figure 5-2. PAI-1$^+$ PEV levels stratified by antiplatelet agent.

(A) Aspirin (ASA) did not affect PAI-1 PAI-1$^+$ PEV levels. (B) P2Y12 inhibitor did not affect PAI-1$^+$ PEV levels. (C) Dual antiplatelet therapy (DAPT) did not affect PAI-1$^+$ PEV levels. No – refers to patients who are not on the respective antiplatelet therapy. Comparison was done by Mann-Whitney U-test. $P<0.05$ was considered statistically significant.

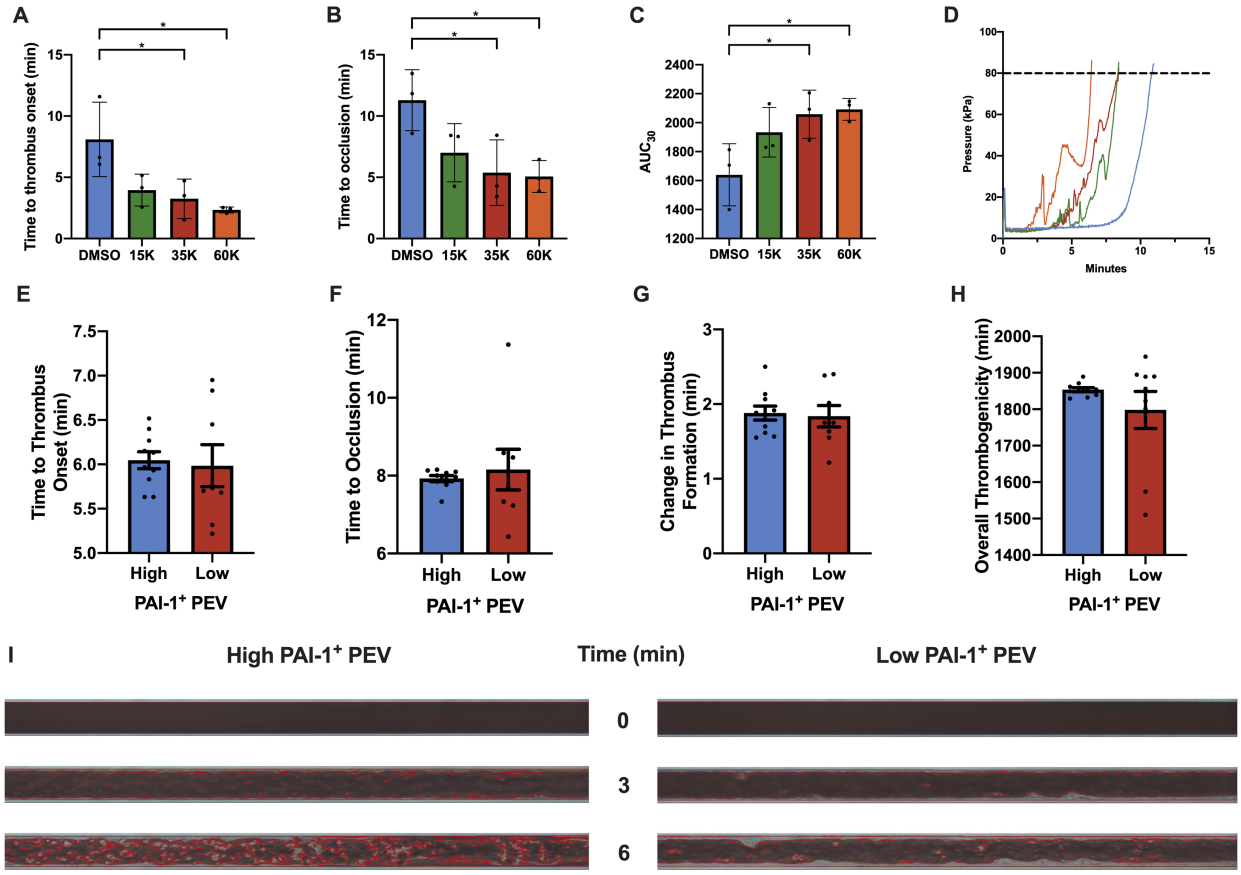


Figure 5-3. Impact of PEV and PAI-1$^+$ PEV on thrombus formation.

(A) Time to onset of thrombus formation (time to reach 10 kPa) decreased with increasing concentrations of PEV (8.1 $\pm$ 3.0 min vs. 4.0 $\pm$ 1.3 min vs. 3.3 $\pm$ 1.6 min vs. 2.3 $\pm$ 0.2 min, for DMSO, 15000, 35000, and 60000 PEV/uL, respectively; n=3). (B) Time to thrombus occlusion (time to reach 80 kPa) decreased with increasing concentrations of PEV (11.3 $\pm$ 2.5 min vs. 7.0 $\pm$ 2.4 min vs. 5.4 $\pm$ 2.7 min vs. 5.1 $\pm$ 1.3 min, for DMSO, 1.5x10^4, 3.5x10^4, and 6.0x10^4 PEV/uL, respectively; n=3). (C) Overall thrombogenicity increased with increasing concentrations of PEV (1640 $\pm$ 214 vs. 1934 $\pm$ 171 vs. 2059 $\pm$ 166 vs. 2092 $\pm$ 75, for DMSO, 1.5x10^4, 3.5x10^4, and 6.0x10^4 PEV/uL, respectively; n=3). (D) Average pressure vs. time curve for DMSO (blue), 1.5x10^4 (green), 3.5x10^4 (red), and 6.0x10^4 PEV/uL (orange). (E) Time to onset of thrombus formation (time to reach 10 kPa) was not different between low and high PAI-1$^+$ PEV fractions (6.0 $\pm$ 0.3 min vs. 6.0 $\pm$ 0.7 min, for high vs. low respectively; p=0.80). (F) Time to thrombus occlusion (time to reach 80 kPa) was not different between low and high PAI-1$^+$ PEV fractions (7.9 $\pm$ 0.2 min vs. 8.2 $\pm$ 1.5 min, for high vs. low respectively; p=0.63). (G) No differences in rate of thrombus formation was observed between low and high PAI-1$^+$ PEV fraction (1.9 $\pm$ 0.3 min vs. 1.8 $\pm$ 0.4 min, for high vs. low respectively; p=0.80). (H) Overall thrombogenicity was not different between low and high PAI-1$^+$ PEV fractions (1854.0 $\pm$ 18.0 AUC vs. 1798.0 $\pm$ 151.8 AUC, for high vs. low respectively; p=0.27). (I) Corresponding microscopic images of thrombus formation of low vs. high PAI-1$^+$ PEV fractions under flow conditions in the AR-Chip.

5.5.3 PAI-1$^+$ PEVs modulate VSMC phenotypic switching

Next, we studied the effect of PAI-1$^+$ PEV on VSMC phenotyping switch using a combination of functional assays and changes in VSMC cellular programming. First, PEV adhesion ($1.0x10^8$/mL) with VSMC incubated in suspension ($5.0x10^5$/mL) was assessed by flow cytometry following staining PEVs with CellTracker CMFDA. The interaction between PEV and VSMC was quantified as an increase in median fluorescence intensity (**MFI**) relative to the unstained VSMC fraction (170.7 ± 98.3 vs. 2.5 ± 0.02; p=0.005; Figure 5-4A). Similarly, co-incubation of CMFDA$^+$ PEVs with VSMCs demonstrates PEV binding in a dose- and time-dependent fashion to VSMCs (Figure 5-5).

We evaluated the effect of PAI-1$^+$ PEV on SMC migration through an *in vitro* scratch assay in the presence of increasing concentrations of PEVs (Figure 5-6). Incubation of SMC with PEVs enhanced wound closure 1.7-fold compared to DMSO control at 24 hours (41.7 ± 24.1%; n=10, 68.5 ± 19.1%; n=6, 69.1% ± 11.5%; n=11, 65.6 ± 9.3%; n=11, and 63.9 ± 15.7%, n=8, for DMSO, PDGF, $1.5x10^4$ PEV/uL, $3.5x10^4$ PEV/uL, and $6.0x10^4$ PEV/uL, respectively, Figure 5-4B, with corresponding images on Figure 5-6). Proliferation was evaluated using CellTrace Violet stained VSMCs at baseline and evaluated by flow cytometry in the presence of increasing concentrations of PEVs. After 24 hours of incubation, only $6.0x10^4$ PEV/uL promoted proliferation compared to DMSO control (4481.0 ± 1179.3 VSMCs vs. 3319.3 ± 863.3 VSMCs, $6.0x10^4$ PEV/uL (n=24) and DMSO (n=7), respectively; p=0.02; Figure 5-4C). After 48 hours of incubation, $3.5x10^4$ PEVs/uL promoted proliferation compared to DMSO control (5473.1 ± 945.4 VSMCs vs. 5658.5 ± 1396.5 VSMCs vs. 4357.9 ± 673.5 VSMCs, for $3.5x10^4$ PEVs/uL (n=24), $6.0x10^4$ PEVs/uL (n=24), and DMSO (n=10), respectively; p=0.004; Figure 5-4D). Finally, VSMC

co-cultured with PEVs exhibited changes in VSMC trans-differentiation markers, KLF4 and ACTA2 (Figure 5-4E).

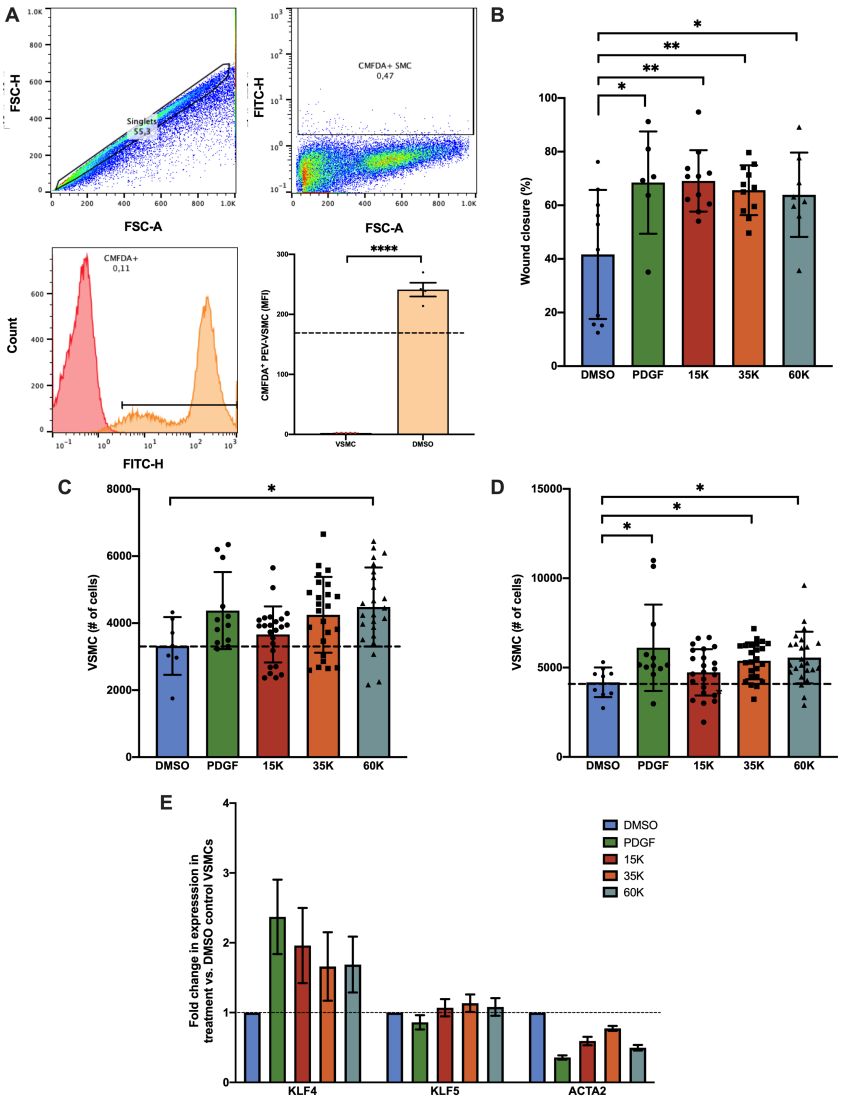


Figure 5-4. PEVs modulate VSMC phenotypic switching.

(A) CMFDA^{+} PEV adhesion is observed in SMCs (170.7 ± 98.3 vs. 2.5 ± **0.02**; p=0.005). (B) Incubation of VSMC with PEVs enhanced migration (wound closure) 1.7-fold compared to DMSO control at 24 hours (41.7 ± 24.1%; n=10, 68.5 ± 19.1%; n=6, 69.1% ± 11.5%; n=11, 65.6 ± 9.3%; n=11, and 63.9 ± 15.7%, n=8, for DMSO, PDGF, 1.5x10^{4}, 3.5x10^{4}, and 6.0x10^{4} PEV/uL, respectively). (C) CellTrace Violet^{+} VSMC proliferation in the presence of increasing concentrations of PEVs at 24 hours. Only 60000 PEV/uL promoted proliferation compared to DMSO control (4481.0 ± 1179.3 VSMCs vs. 3319.3 ± 863.3 VSMCs, 6.0x10^{4} PEV/uL (n=24) and DMSO (n=7), respectively; p=0.02). (D) CellTrace Violet^{+} VSMC proliferation in the presence of increasing concentrations of PEVs at 48 hours. Both 3.5x10^{4} and 6.0x10^{4} PEVs/uL promoted proliferation compared to DMSO control (5473.1 ± 945.4 VSMCs vs. 5658.5 ± 1396.5 VSMCs vs. 4357.9 ± 673.5 VSMCs, for 35000 PEVs/uL (n=24), 6.0x10^{4} PEVs/uL (n=24), and DMSO (n=10), respectively; p=0.004). (D) VSMC co-cultured with PEVs exhibited changes in VSMC differentiation markers, KLF4 and ACTA2. P<0.05 was considered statistically significant. VSMC = smooth muscle cell; 15K, 35K, and 60K represent 1.5x10^{4}, 3.5x10^{4}, and 6.0x10^{4} PEV/uL.

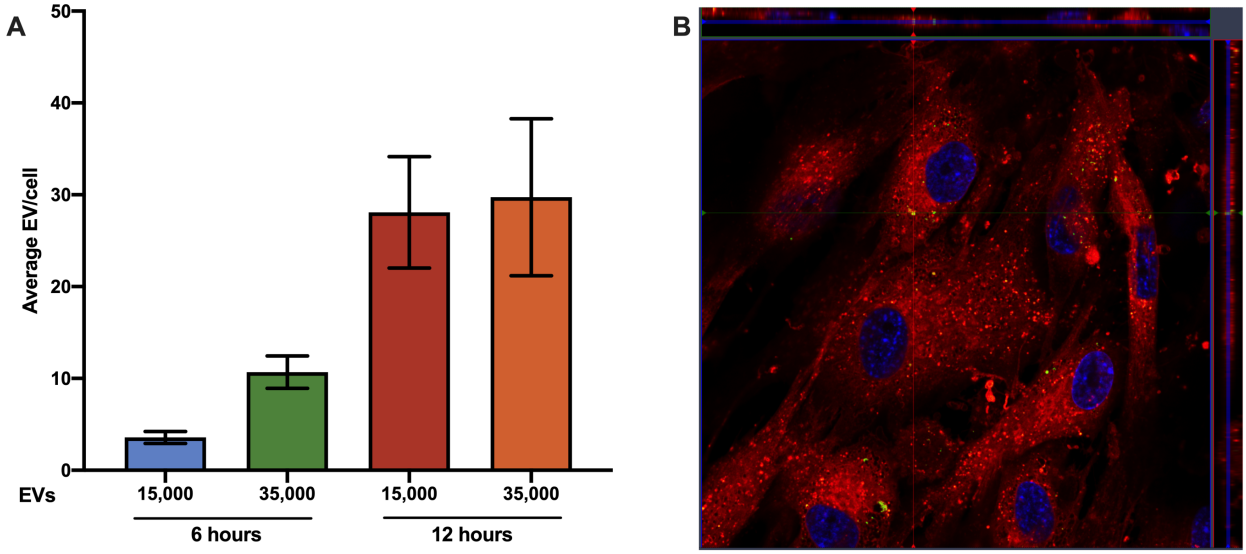


Figure 5-5. Interaction between PEV and VSMCs.

(A) Incubation with increasing concentrations of $CMFDA^{+}$ PEV with VSMCs assessed by flow cytometry reveals increased interaction in a dose- and time- dependent fashion. (B) Representative immunofluorescence image to evaluate PEV-VSMC interaction. PEV were stained using CellTracker Green (arrow), VSMC was stained in DAPI and CellMask Orange.

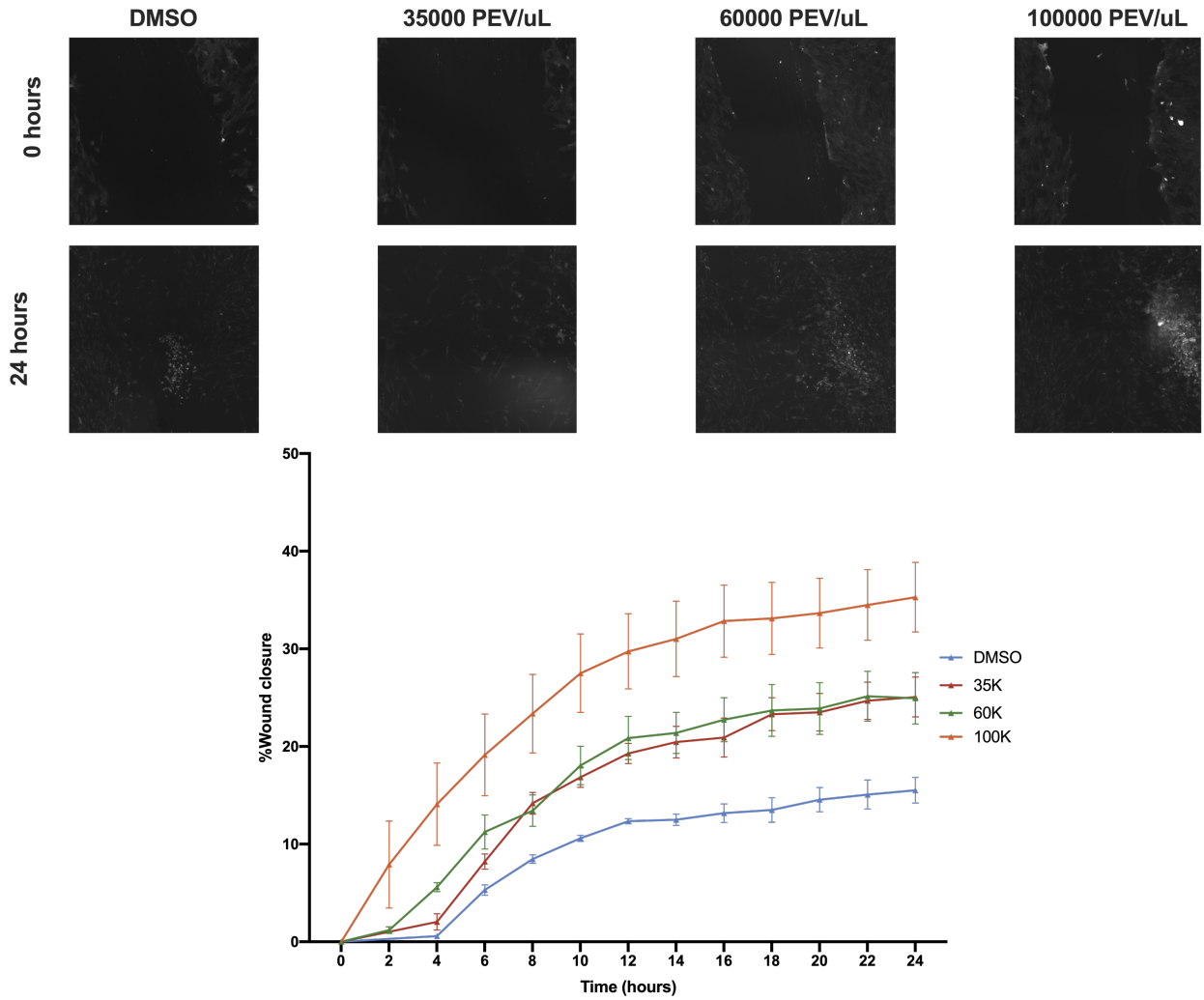


Figure 5-6. Effect of PEV on VSMC migration by scratch assay.

Top panels reveal corresponding microscopy images from the initial scratch at 0 hours to 24 hours. Increasing concentrations of PEV (DMSO, 35000, 60000, and 100000 EVs/uL) enhanced VSMC migration over 24 hours as measured using the Cytation 5.

5.5.4 PAI-1$^+$ PEV is elevated in major adverse cardiac events

The baseline characteristics of 456 patients who had one-year clinical follow-up with enumerated PAI-1$^+$ PEVs is found in Table 5-1. Briefly, the mean age of the cohort was 66.7 $\pm$ 11.3 years and 124 patients (27.2%) were female. Importantly, patients had mixed indications for angiography, including both stable CAD (36.2%) and acute coronary syndrome (44.5%), and 270 patients (59.2%) underwent revascularization in the form of PCI or coronary artery bypass grafting. Finally, 357 patients (78.3%) had $\geq$1 vessel-burden with significant CAD.

During a median follow-up period of 12.7 months (IQR, 10.6 to 15.2 months), 76 patients (16.7%) in our cohort experienced MACE with 36 deaths, 7 myocardial infarctions, 28 unplanned revascularizations, and 18 cerebrovascular accidents. When stratified by MACE, differences in rates of previous PCI and MI was observed (Table 5-2). Patients who had MACE had significantly elevated PAI-1$^+$ PEV levels (13786.6 [6711.7-26942.1] PAI-1$^+$ PEV/uL vs. 8344.9 [3777.2-33499.7] PAI-1$^+$ PEV/uL, n=76 and 380, respectively, p=0.047, Figure 5-7A) and elevated PEV levels (107463.0 [58416.1-202987.8] PEV/uL vs. 71675.6 [31184.5-201313.3] PEV/uL, n=76 and 380, respectively, p=0.04, Figure 5-7B). Furthermore, no differences were observed in plasma PAI-1 levels and (PAI-1$^+$ PEV)/PEV fraction between event and no-event (Figure 5-7C-D).

Table 5-1. Baseline characteristics

	Total (n=456)
Age - mean (SD)	66.7 (11.3)
Sex (female) - no. (%)	124 (27.2)
Hypertension - no. (%)	302 (66.2)
Dyslipidemia - no. (%)	277 (60.8)
Diabetes - no. (%)	147 (32.2)
Smoking - no. (%)	
Never	265 (58.1)
Remote (quit >1 month ago)	112 (24.6)
Active	79 (17.3)
Family history of CAD - no. (%)	65 (14.3)
Atrial fibrillation - no. (%)	48 (10.5)
Indications for angiography - no. (%)	
Acute coronary syndrome	203 (44.5)
Staged percutaneous coronary intervention	42 (9.2)
Stable coronary artery disease	165 (36.2)
Shock	1 (0.2)
Previous history - no. (%)	
Percutaneous coronary intervention	118 (25.9)
Myocardial infarction	85 (18.6)
Coronary artery bypass grafting	23 (5.0)
Peripheral artery disease	36 (7.9)
Cerebrovascular accident	27 (5.9)
Bleed	8 (1.8)
Heart failure	23 (5.0)
Medications - no. (%)	
Aspirin	415 (91.0)
P2Y12 inhibitor	410 (89.9)
ACEi/ARB	238 (52.2)
Beta blocker	268 (58.8)
Calcium channel blocker	63 (13.8)
Statin	372 (81.6)
Proton pump inhibitor	71 (15.6)
Number of vessels with obstructive (≥ 50%) CAD - no. (%)	
0	99 (21.7)
1	193 (42.3)
2	164 (36.0)
Revascularized (PCI + CABG) - no. (%)	270 (59.2)

Table 5-2. Differences in baseline characteristics by major adverse cardiac events

	No MACE (n=380)	MACE (n=76)	P-value
Age - mean (SD)	65.5 (11.1)	72.5 (10.9)	<0.0001
Sex (female) - no. (%)	100 (26.3)	24 (31.6)	0.35
Hypertension - no. (%)	250 (65.8)	52 (68.4)	0.66
Dyslipidemia - no. (%)	221 (58.2)	56 (73.7)	0.01
Diabetes - no. (%)	112 (29.9)	35 (46.1)	0.006
Smoking - no. (%)			0.37
Never	226 (59.5)	39 (51.3)	
Remote (quit >1 month ago)	89 (23.4)	23 (30.3)	
Active	65 (17.1)	14 (18.4)	
Family history of CAD - no. (%)	55 (14.5)	10 (13.2)	0.76
Atrial fibrillation - no. (%)	37 (9.7)	11 (14.5)	0.22
Indications for angiography - no. (%)			
Acute coronary syndrome	165 (43.4)	38 (50.0)	0.29
Staged percutaneous coronary intervention	32 (8.4)	10 (13.2)	0.19
Stable coronary artery disease	144 (37.9)	21 (27.6)	0.09
Shock	1 (0.3)	0 (0.0)	0.65
Previous history - no. (%)			
Percutaneous coronary intervention	96 (25.3)	22 (29.0)	0.5
Myocardial infarction	63 (16.6)	22 (29.0)	0.01
Coronary artery bypass grafting	14 (3.7)	9 (11.8)	0.003
Peripheral artery disease	25 (6.6)	11 (14.5)	0.02
Cerebrovascular accident	19 (5.0)	9 (10.5)	0.06
Bleed	7 (1.8)	1 (1.3)	0.75
Heart failure	19 (5.0)	4 (5.3)	0.92
Medications - no. (%)			
Aspirin	346 (91.1)	69 (90.8)	0.94
P2Y12 inhibitor	343 (90.3)	67 (88.2)	0.58
ACEi/ARB	199 (52.4)	39 (51.3)	0.87
Beta blocker	223 (58.7)	45 (59.2)	0.93
Calcium channel blocker	48 (12.6)	15 (19.7)	0.1
Statin	307 (80.8)	54 (85.5)	0.33
Proton pump inhibitor	56 (14.7)	15 (19.7)	0.27
Number of vessels with obstructive (≥ 50%)			
CAD - no. (%)			0.49
0	86 (22.6)	13 (17.1)	
1	157 (41.3)	36 (47.4)	
2	137 (36.1)	27 (35.5)	
Revascularized (PCI + CABG) - no. (%)	235 (61.8)	35 (46.1)	0.01

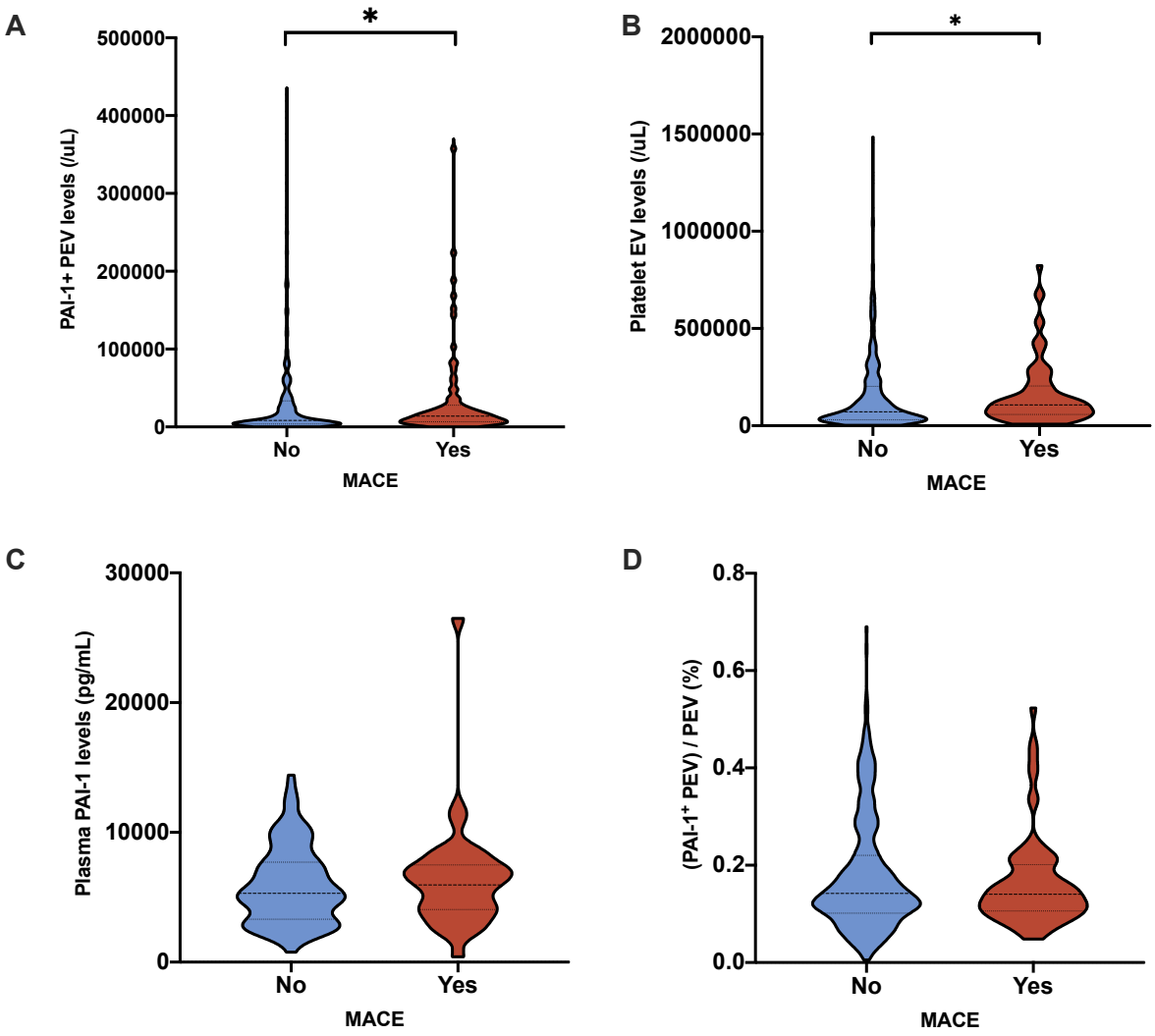


Figure 5-7. Difference in PEV levels (/uL) stratified by MACE.

(A) PAI-1$^+$ PEV levels were significantly elevated in MACE (13786.6 [6711.7-26942.1] PAI-1$^+$ PEV/uL vs. 8344.9 [3777.2-33499.7] PAI-1$^+$ PEV/uL, n=76 and 380, respectively, p=0.047). (B) PEV levels were elevated in MACE (107463.0 [58416.1-202987.8] PEV/uL vs. 71675.6 [31184.5-201313.3] PEV/uL, n=76 and 380, respectively, p=0.04). (C) No differences in plasma PAI-1 levels were observed when stratified by MACE. (D) No differences in PAI-1$^+$ PEV fraction was observed when stratified by MACE. Data is presented as bar graph and log-transformed data was compared by student t-test. P<0.05 is considered statistically significant. *P<0.05.

5.5.5 Utility of PAI-1$^+$ PEV as a biomarker of MACE: discovery and validation cohort

In an all-comer population, we sought to evaluate whether PAI-1$^+$ PEV levels were predictive of MACE and the cohort was equally divided into a discovery and validation cohort. The optimal cut-off point (Youden's Index) identified in the discovery cohort for prediction of MACE for PAI-1$^+$ PEV was ≥6321.9/uL with an area under the curve (**AUC**) of 0.60 (95% CI, 0.55-0.65) and a crude MACE rate of 262.3 vs. 103.0 events per 1,000 person-years for high vs. low PAI-1$^+$ PEV levels, respectively. High PAI-1$^+$ PEV levels were associated with MACE with a HR of 2.43 (95% CI, 1.43 to 4.13, p=0.001, Table 5-3**,** Model 1). When adjusted for clinical variables associated with MACE (age, DM, gender, and acute coronary syndrome), high PAI-1$^+$ PEV levels remained associated with MACE with a HR of 2.49 (95% CI, 1.46 to 4.25, p=0.0008) and PAI-1$^+$ PEV was the strongest predictor of MACE compared to the best-known clinical variables (Table 5-3, Model 2). In the discovery cohort, high PAI-1$^+$ PEV levels were associated with MACE with a HR of 2.19 (95% CI, 1.07 to 4.52, log-rank p=0.03, Figure 5-8A). In the validation cohort, high PAI-1$^+$ PEV levels remained predictive of MACE with a HR of 2.67 (95% CI, 1.22 to 5.84, log-rank p=0.01, Figure 5-8B).

Table 5-3. Major adverse cardiac events predicted by PAI-1$^+$ PEV levels

Model for MACE	PAI-1$^+$ PEV HR (95% CI)	P-value
Model 1 - unadjusted PAI-1 PEV levels		
PAI-1$^+$ PEV cutoff at Youden's point	2.43 (1.43-4.13)	0.001
Model 2 - adjusted by age (years), acute coronary syndrome, sex, and type 2 diabetes		
PAI-1$^+$ PEV cutoff at Youden's point	2.49 (1.46-4.25)	0.0008
Age (years)	1.06 (1.04-1.08)	<0.0001
Acute coronary syndrome	1.19 (0.75-1.89)	0.46
Sex	1.07 (0.65-1.75)	0.80
Type 2 diabetes	1.75 (1.11-2.76)	0.02

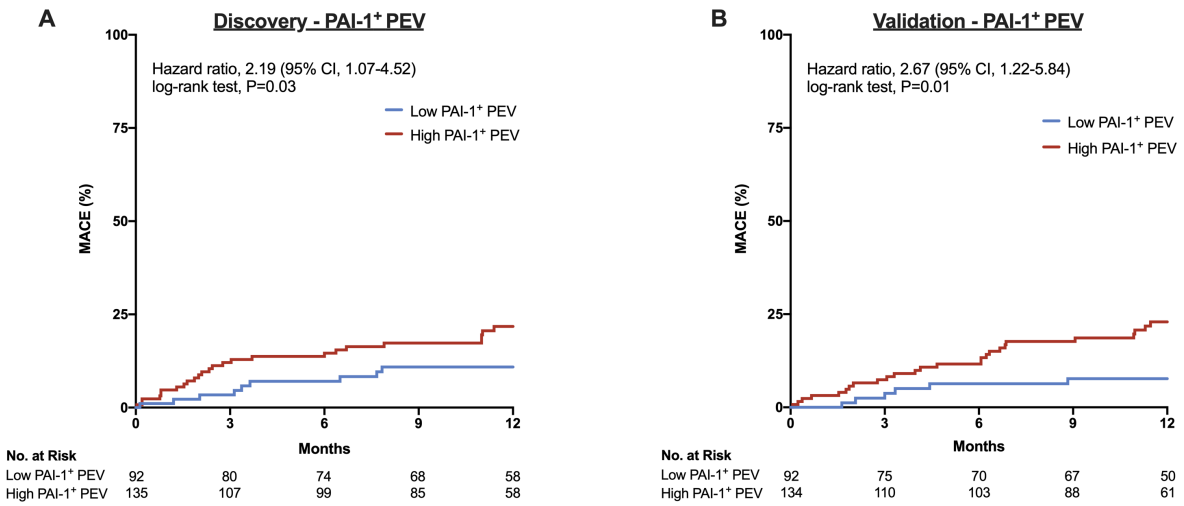


Figure 5-8. Utility of PAI-1$^+$ PEV complex for prediction of MACE.

(A) In the discovery cohort, patients with cumulative incidence of MACE within one-year follow-up, high PAI-1$^+$ PEV levels were predictive of MACE (hazard ratio, 2.19; 95% CI, 1.07 to 4.52, log-rank p=0.03). (B) In the validation cohort, patients with cumulative incidence of MACE within one-year follow-up, high PAI-1$^+$ PEV levels remained predictive of MACE (hazard ratio, 2.67; 95% CI, 1.22 to 5.84, log-rank p=0.01). Kaplan-Meier curves were generated and compared by log-rank test and hazard ratios were evaluated using the Cox proportional hazards model. $P<0.05$ is considered statistically significant.

5.5.6 Inhibition of PAI-1 attenuates SMC phenotypic switch but not thrombus formation

Finally, the functional effect of PAI-1 on PAI-1$^+$ PEV was evaluated using two known PAI-1 inhibitors: TM5275 (inhibits PAI-1 and LRP1 interaction) and tiplaxtinin (inhibits PAI-1 by binding to vitronectin-binding site) after validating PAI-1 activity on the surface of PAI-1$^+$ PEVs (Figure 5-9). Neither of the two inhibitors affected thrombus formation in T-TAS with no change in onset of thrombus formation, occlusion, nor rate of thrombus formation, which not ameliorate the overall thrombogenicity (Figure 5-10A-D). The addition of TM5275 reduced CMFDA$^+$ PEV and VSMC interaction by 3.7-fold (62.5 $\pm$ 20.6 MFI vs. 241.3 $\pm$ 22.9 MFI; p=0.0008; Figure 5-10E) whereas tiplaxtinin had no effect on PEV-VSMC interaction (167.1 $\pm$ 92.4 vs. 241.3 $\pm$ 22.9; p=0.25). TM5275 significantly reduced VSMC proliferation regardless of the PEV dosage ($1.5x10^4$ or $3.5x10^4$ PEV/uL) at both 24 and 48-hour timepoints (Figure 5-10F-G).

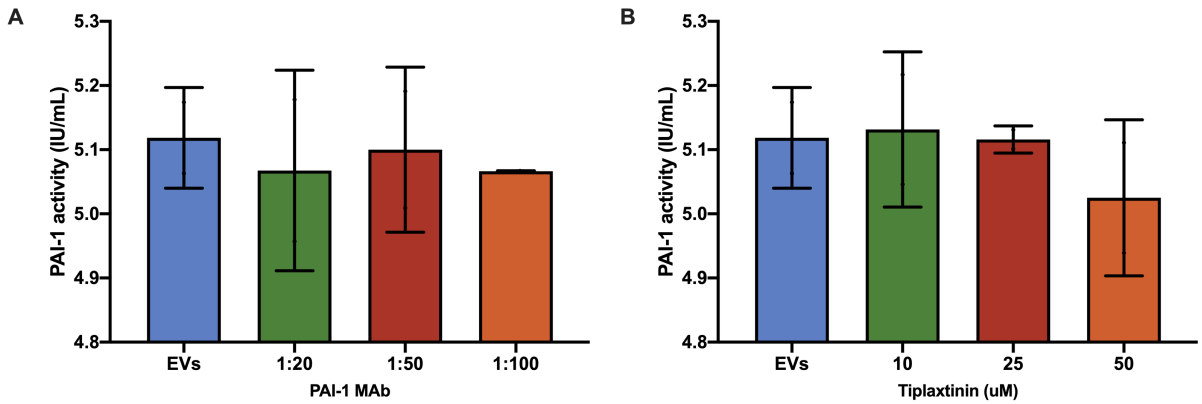


Figure 5-9. PAI-1 activity of PAI-1$^+$ PEV complex in the presence of PAI-1 inhibition.

(A) The addition of PAI-1 monoclonal antibody (MAb) had no effect on activity of PAI-1$^+$ PEV complex (IU/mL). (B) Increasing concentrations of tiplaxtinin had no effect on PAI-1$^+$ PEV complex activity. $P<0.05$ is considered statistically significant.

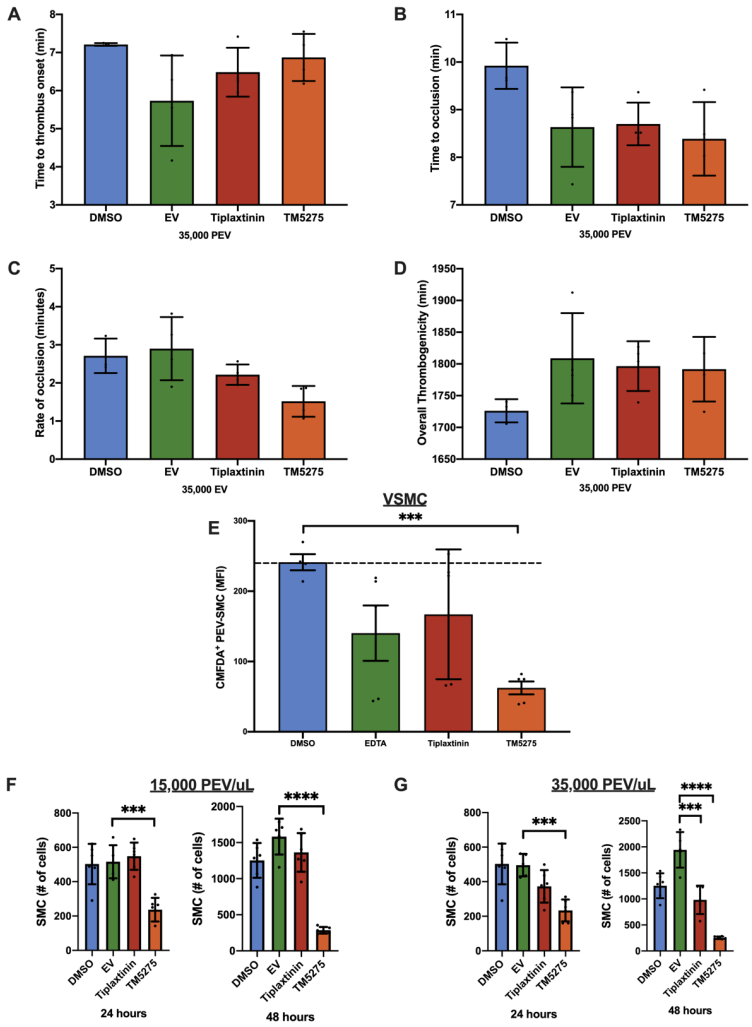


Figure 5-10. Inhibition of PAI-1 does not affect thrombus formation but attenuates the pro-inflammatory VSMC phenotype.

(A) No difference in time to onset of thrombus formation (time to reach 10 kPa) was observed in the presence of PAI-1 inhibitors (5.7 ± 1.2 min vs. 6.5 ± 0.6 min vs. 6.9 ± 0.6 min for EV, 25 μM tiplaxtinin, and 0.1 μM TM5275, respectively; n=4; p=0.80). (B) Time to thrombus occlusion (time to reach 80 kPa) was not different in the presence of PAI-1 inhibitors (8.6 ± 0.8 min vs. 8.7 ± 0.4 min vs. 8.4 ± 0.8 min, for EV, 25 μM tiplaxtinin, and 0.1 μM TM5275, respectively; n=4; p=0.63). (C) No differences in rate of thrombus formation was observed in the presence of PAI-1 inhibitors (2.9 ± 0.8 min vs. 2.2 ± 0.3 min vs. 1.5 ± 0.4 min, for EV, 25 μM tiplaxtinin, and 0.1 μM TM5275, respectively; n=4; p=0.80). (D) Overall thrombogenicity was not different in the presence of PAI-1 inhibitors (1809 ± 71 AUC vs. 1797 ± 39 AUC vs. 1792 ± 51 AUC, for EV, 25 μM tiplaxtinin, and 0.1 μM TM5275, respectively; n=4; p=0.27). (E) $CMFDA^{+}$ PEV and VSMC interaction was attenuated in the presence of 0.1 μM TM5275 (62.5 ± 20.6 MFI vs. 241.3 ± 22.9 MFI; n=5; p=0.0008) but not with 25 μM tiplaxtinin (167.1 ± 92.4 vs. 241.3 ± 22.9; n=5; p=0.25). (F) VSMC proliferation was inhibited in the presence of TM5275 after 24- and 48-hour incubation of 15,000 PEV/μL. (G) VSMC proliferation was inhibited in the presence of TM5275 after 24- and 48-hour incubation of $3.5x10^4$ PEV/μL.

5.6 Discussion

Patients with CAD continue to accrue adverse events despite guideline-directed medical therapy and revascularization. Identification of novel markers to risk stratify patients and/or to offer new therapeutic interventions remains a priority. Given the potential of EVs to behave as a biomarker and due to the common origin of PAI-1 and EV from platelets, our study sought to evaluate whether PAI-1^+ PEV predicts MACE and affects thrombus formation and pro-inflammatory VSMC phenotype. Our study presents five novels findings regarding PAI-1^+ PEV: 1) the presence of a PAI-1^+ PEV complex in human plasma, 2) PAI-1^+ PEVs in standardized PEV fraction has no effect on thrombus formation, 3) PEV promotes the pro-inflammatory VSMC phenotype, 4) inhibition of PAI-1^+ PEV through TM5275 attenuates VSMC phenotypic switching but has no effect on thrombus formation, and 5) high PAI-1^+ PEV levels were predictive of MACE in both the discovery and validation cohort. Overall, PAI-1^+ PEV acts as a promising biomarker and offers a novel target to modulate vessel biology in patients with CAD.

We hypothesized that circulating plasma PAI-1 was largely EV derived and would modulate event rates in patients with CAD by increasing thrombotic risk. We found that 19.1% of circulating PAI-1 is in PAI-1^+ PEVs – a newly identified subgroup of circulating PEVs. In our *in vitro* models, PAI-1^+ PEVs did not increase thrombogenicity compared to PEVs alone. Interestingly, PEVs were demonstrated to interact with VSMCs, subsequently increasing phenotypic switching, migration, and synthetic function. Inhibition models confirmed VSMCs sequestered PEVs through a PAI-1 and LRP-1 dependent interaction resulting in VSMC changes. Finally, high levels of circulating PAI-1^+ PEV was strongly associated with adverse clinical outcomes in our cohort suggesting a strong potential as a biomarker of adverse events in high-risk patients.

PAI-1 mainly functions as an anti-proteolytic protein but also appears to serve a ligand role affecting migration, proliferation, and apoptosis at vascular sites of injury.[75, 76, 247, 248] In our study, we demonstrate that increasing concentrations of PAI-1$^+$ PEV drives the pathologic changes observed in pro-inflammatory VSMCs to drive neointimal formation. Specifically, PAI-1 was previously shown to interact with LRP1 to stimulate the JAK/STAT-1 signaling cascade, driving VSMC migration.[76] Indeed, PAI-1 inhibition with TM5275, an inhibitor of PAI-1 and LRP1 interaction, greatly attenuated PEV-VSMC interaction and VSMC proliferation in our model, whereas tiplaxtinin had no effect. This observation is crucial as TM5275 inhibits PAI-1 interaction with LRP1, whereas tiplaxtinin inhibits PAI-1 by binding to its vitronectin-binding site.[249] Although we do not understand how PAI-1 remains bound on the surface of PEVs, we hypothesize that a complex formed between vitronectin (and its associated integrin receptor) with PAI-1 protects it from tiplaxtinin to serve as a signaling ligand of the PAI-1$^+$ PEV complex.[250] Indeed, the effect of PAI-1$^+$ PEV modulation on the pro-inflammatory VSMC phenotype was attenuated with TM5275 and is a promising therapeutic target to reduce neointimal formation.

PEVs functions in thrombosis with coagulation factors or phosphatidylserine exposure present on the surface to mediate coagulation.[251] The presence of active PAI-1 on the surface of PEVs potentially mediates thrombosis by inhibiting the fibrinolytic cascade. Furthermore, circulating EVs have previously been demonstrated to mediate thrombosis by binding to the subcellular matrix following endothelial injury.[252] We have demonstrated that increased concentrations of PEVs drives thrombus formation. However, when standardized amount of PEVs with differing PAI-1$^+$ PEV content were added, no differences in thrombus onset nor occlusion was observed. Furthermore, the addition of PAI-1 inhibitors had no effect on thrombus formation nor thrombogenicity. Interestingly, no association between DAPT and PAI-1$^+$ PEV levels was

observed, potentially confounded by the observation that >90% of the cohort was administered aspirin at the time of catheterization. This suggests that the overall procoagulant activity on PAI-1^+ PEV is not primarily mediated by PAI-1 but is likely mediated by other surface factors such as tissue factor and factor VII. This finding confirms the lack of mediatory role of PAI-1 with ischemic events.[203]

Finally, our results demonstrate that elevated levels of PAI-1^+ PEV were strongly predictive of MACE out to 1-year. Our study adds to the existing literature in that EVs have strong utility to predict cardiovascular events and are promising targets as they are the major hub linking inflammation, vascular injury, coagulation, and cellular reprogramming responsible for both acute and chronic complications.[237, 253-255] In our current study, PAI-1^+ PEV performed remarkably with a very large predictive effect with hazard ratios of 2.19 and 2.67 in the discovery and validation cohort respectively. Comparatively, the effect size outperforms the most potent biomarkers in CAD such as troponin (HR 1.47 in 10-year follow-up), coronary artery calcium (HR 2.35 in 10-year follow-up), age (HR 1.04 in 3-year follow-up), BNP (HR 1.24 in 3-year follow-up), and hsCRP (HR 1.47 in 3-year follow-up).[256, 257] Moreover, our dataset now stands as the largest evaluation to date of EVs as a biomarker. Comparatively, the largest EV biomarker study to date prior to the current study was in 200 stable CAD patients demonstrating the prognostic value of $CD31^+$/Annexin V^+ EVs for MACE in six-years follow-up with a hazard ratio of 4.0.[258] Previous biomarker studies have reported elevated levels of EVs with acute coronary syndrome and end-stage renal disease.[228, 256, 259, 260] The totality of the data suggest that EVs may serve an unmet clinical need providing robust risk stratification – with PAI-1^+ PEVs demonstrating the most impactful risk stratification to date.

This study is not without its limitations. First, being a highly predictive biomarker of adverse events in of itself does not confer causal inferences and we relied on *in vitro* models to assess the effect of EVs on thrombosis and VSMC biology respectively. Second, we are not able to isolate PAI-1$^+$ PEV fraction from PEVs without inhibiting PAI-1. Furthermore, all bench-top experiments were performed using EVs isolated from healthy volunteers. Accordingly, we were not able to directly observe the effect of the complex on VSMC phenotypic switching. Finally, the effect of PAI-1$^+$ PEV on VSMC phenotypic switching is not testable in an *in vivo* study. To date, animal models do not exist where we can manipulate EV production or exogenously administer EVs without initiating the host inflammatory response. Therapeutic development of these observations will likely rely on PAI-1 inhibition – a strategy previously demonstrated to be efficacious in mouse models of arterial injury.[261, 262]

5.7 Conclusion

In conclusion, we report the existence of a PAI-1$^+$ PEV complex in humans that functions as a modulator of pro-inflammatory changes of neointimal formation in smooth muscle cells. Moreover, PAI-1$^+$ PEVs can be used as a powerful biomarker to identify high-risk patients with CAD. Finally, given the attenuation of VSMC phenotypic switching through the inhibition of the PAI-1/LRP1 response, our study suggests PAI-1 as a potential biomarker to identify and a therapeutic target to ameliorate neointimal formation of high-risk patients.

5.8 Sources of Funding

The authors would like the CAPITAL Research Group for their assistance to complete the project. This project was supported by the Innovation Fund of the Alternative Funding Plan for the Academic Health Sciences Centres of Ontario and Canadian Foundation of Innovation. R.G.J. was

funded by the Vanier Canadian Institutes of Health Research (CIHR) Canada Graduate Scholarship for his graduate studies. A.C.D. was supported by the Cardiac Endowment Fund at the University of Ottawa Heart Institute. T.S. was supported by the Vered-Beanlands Endowed Fellowship at the University of Ottawa Heart Institute. S.R. was supported by CIHR Canada Graduate Scholarship for her Masters' studies.

5.9 Conflict of interest

The authors declare that they have no competing interests.

Chapter 6

Discussion

6.1 Summary of Findings

PAI-1 has previously been associated with MACE, but whether it is solely a marker for these events or a mediator with the potential of representing a unique therapeutic target is uncertain.[25, 112, 154, 176, 178, 179] Our systematic review and meta-analysis (Chapter 2) suggests that elevated PAI-1 antigen levels are associated with major adverse cardiac events in both primary and secondary event populations. In addition, elevated PAI-1 antigen levels were associated with all-cause mortality.[205]

Despite numerous biomarker studies of PAI-1 conducted in the past, there remains a paucity of analytical and biochemical performance of PAI-1 as a biomarker in the literature. We analyzed 689 patients who underwent coronary angiography at the University of Ottawa Heart Institute and identified that plasma PAI-1 has similar analytical performance characteristics to clinically employed biomarkers such as CRP and NT-proBNP which have high CV_g and CV_i. Moreover, due to a low index of individuality of PAI-1 (II <0.6), changes from population-based reference values in an individual have limited value as a diagnostic test to detect pathologies but may be useful as a monitoring test in patients undergoing coronary angiography.[190, 194] The measured critical difference (at $P \leq 0.05$) between two serial measurements of PAI-1 was determined to be 122%, similar in performance to CRP (118%).[192]

Next, we set out to evaluate the role of plasma PAI-1 as a biomarker of one-year major adverse cardiac events and unplanned revascularization following coronary angiography. Plasma PAI-1 was not found to be an independent predictor of UR nor of MACE but was associated with

UR after adjustment for known clinical factors. Moreover, in an updated systematic review and meta-analysis including our dataset, plasma PAI-1 was not predictive of MACE in either the all-comer or PCI-only population. Interestingly, a *post-hoc* landmark analysis beyond the peri-procedural period (one-month) suggested that low plasma PAI-1 levels were associated with stent complications.

The last chapter of the thesis focuses on the role of PAI-1$^+$ PEV as a biomarker of MACE and mechanistic insights of PAI-1$^+$ PEV driving in-stent restenosis and stent thrombosis. As previously alluded, as both plasma PAI-1 and extracellular vesicles predominantly originate from circulating platelets, we hypothesized that PAI-1$^+$ PEV represents a major source of pathologic PAI-1. This study identified five novel findings: 1) the presence of a PAI-1$^+$ PEV complex in human plasma, 2) PAI-1$^+$ PEVs in standardized PEV fraction has no effect on thrombus formation, 3) PEV promotes the pro-inflammatory VSMC phenotype, 4) inhibition of PAI-1$^+$ PEV through TM5275 attenuates VSMC phenotypic switching but has no effect on thrombus formation, and 5) high PAI-1$^+$ PEV levels were predictive of MACE in both the discovery and validation cohort.

6.2 Implication of PAI-1 as a biomarker following revascularization

In our angiography-based study, the lack of association between plasma PAI-1 and MACE stands in contrast to previously published work.[25, 202, 210] Compared to the current study in the thesis, these studies were conducted in populations without pre-existing cardiovascular disease whereas our cohort consists of patients with a high pre-test probability of CAD presenting for revascularization. Our *post-hoc* landmark analysis suggested that PAI-1 was not associated with unplanned revascularization in the acute post-procedural period but was predictive of late complications in the period where ISR remains predominant.

In our cohort, plasma PAI-1 levels were associated with unplanned revascularization following adjustment with clinical variables. Previous observational studies demonstrated that low levels of plasma PAI-1 were associated with ISR following PCI.[180, 220-222] Contemporary molecular understanding of in-stent restenosis is believed to be driven by the recruitment of inflammatory cells beyond the peri-procedural period of one-month, driving vascular SMC phenotypic switching to a hyper-proliferative and migratory state to narrow and eventually obstruct the vascular lumen to cause ISR.[108] Patients in our study with unplanned revascularization experienced stent failure and the measurement of plasma PAI-1 at the time of catheterization potentially has the additive effect of identifying high-risk populations following PCI since no established risk scores yet exists.

We then sought out to evaluate the role of PAI-1$^+$ PEV as a biomarker of MACE. The rationale for evaluating extracellular vesicles occurred as we sought to determine plausible mechanisms of action of PAI-1 mediating ISR. Moreover, given the understanding that both circulating PAI-1 and extracellular vesicles predominantly originate from platelets in the vasculature, we wished to evaluate whether PAI-1 and platelet EVs exist as a complex and whether they predict MACE and mediate stent failure.[68, 253] We identified that 19.1% of circulating PAI-1 is in the form of PAI-1$^+$ PEV, and this complex was strongly predictive of one-year MACE (**Chapter 5 – Figure 1-8**). The effect size outperforms biomarkers in CAD including troponin (HR 1.47 in 10-year follow-up), coronary artery calcium (HR 2.35 in 10-year follow-up), age (HR 1.04 in 3-year follow-up), BNP (HR 1.24 in 3-year follow-up), and hsCRP (HR 1.47 in 3-year follow-up).[256, 257] This was also the largest evaluation of EV as a biomarker in CAD, and the totality of the data suggest that EVs may serve an unmet clinical need providing robust risk stratification – with PAI-1$^+$ PEVs demonstrating the most impactful risk stratification to date.

6.3 Mechanism of PAI-1 platelet-derived extracellular vesicles on ISR and ST

PAI-1 mainly functions as an anti-proteolytic protein but also appears to serve a ligand role affecting migration, proliferation, and apoptosis at vascular sites of injury.[75, 76, 247, 248] Interestingly, PAI-1 has previously been demonstrated to both promote or inhibit cell migration depending on the animal and tissue culture model used for the experiments (**Chapter 1 – Figure 2**). Briefly, we know that PAI-1 may promote cellular proliferation and migration by binding to LRP1 and activating the JAK/STAT pathway in VSMCs.[76] Binding of PAI-1 to LRP1 appears to affect cell proliferation and migration, and further exploration of PAI-1 binding to LRP1 in mouse embryonic fibroblasts (MEFs).[77]

In Chapter 5, we show that increasing concentrations of PAI-1^+ PEV drives the pathologic changes observed in pro-inflammatory VSMCs to drive neointimal formation. Specifically, PAI-1 inhibition with TM5275, an inhibitor of PAI-1 and LRP1 interaction, greatly attenuated PEV-VSMC interaction and VSMC proliferation in our model, whereas tiplaxtinin had no effect. This observation is crucial as TM5275 inhibits PAI-1 interaction with LRP1, whereas tiplaxtinin inhibits PAI-1 by binding to its vitronectin-binding site.[249] The effect of PAI-1^+ PEV modulation on the pro-inflammatory VSMC phenotype was attenuated with TM5275 and is a promising therapeutic target to reduce neointimal formation.

Stent thrombosis is mediated by thrombus formation within the stented lumen subsequently causing occlusion and myocardial infarction. ST occurs predominantly due to three reasons: (i) Incomplete stent apposition to the coronary lesion; (ii) Incomplete re-endothelialization and delayed healing of the stented vessel; (iii) inadequate antiplatelet administration.[1] As PAI-1 primarily functions as an anti-fibrinolytic protein, elevated levels have been associated in both myocardial infarction and stroke.[79]

Indeed, the potential mechanism between PAI-1^{+} PEV and VSMCs hinges on an interaction between PAI-1 and LRP1, resulting in the downstream activation of beta-catenin or JAK/STAT pathway[203]. We postulate that TM5275 is an effective inhibitor of PAI-1:LRP1 interaction, whereas Tiplaxtinin does not inhibit this interaction as its binding site is bound by vitronectin; however, we do not understand the receptor present on the surface of extracellular vesicles that mediates the response (Figure 6-1).

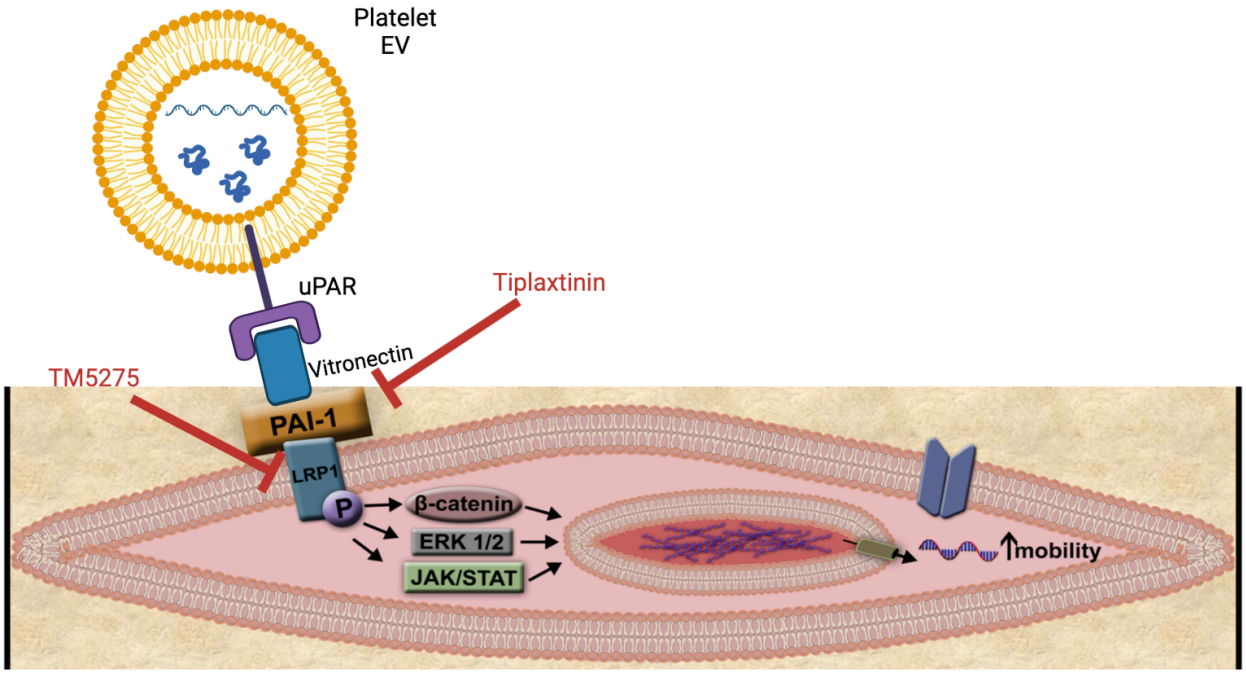


Figure 6-1. Mechanism of action of PAI-1$^+$ PEV and VSMCs.

PAI-1 bound to vitronectin interacts with LRP-1 on the surface of vascular smooth muscle cells to mediate SMC proliferation and migration through the beta-catenin or JAK/STAT pathway. This effect is attenuated with the addition of TM5275, but not with tiplaxtinin.

Our study confirms previous findings that EVs mediates thrombosis due to coagulation factors and phosphatidylserine present on the surface.[251] Moreover, it was hypothesized that active PAI-1 present on the surface of EVs would mediate thrombosis. When a standardized concentration of PEVs with different PAI-1$^+$ PEV fraction was added on a bench-top thrombosis model, no differences in thrombus onset nor occlusion was observed. Furthermore, the addition of PAI-1 inhibitors had no effect on thrombus formation. Thus, the overall procoagulant activity on PAI-1$^+$ PEV appears to not be mediated by PAI-1 but is likely driven by other surface factors such as tissue factor and factor VII.

6.4 Limitations of experimental studies and barriers to clinical translation

This thesis is not without its limitations. Plasma PAI-1 was measured from a single timepoint in patients present for coronary angiography. Moreover, PAI-1 is influenced by circadian rhythm, which was not accurately captured by accounting for daytime variation of blood draws. [85] This was also a retrospective observational study and plasma PAI-1 levels may potentially be influenced by confounding variables captured in the statistical analysis. Our biomarker studies would benefit from prospective study design but remains limited by the experimental methods and time-constraints. Processing both plasma PAI-1 and PAI-1^+ PEV levels takes roughly six hours, whereas patients scheduled for day-time procedures are often discharged within four hours following the completion of their revascularization procedure.

For our bench-top experiments, we relied on *in vitro* models to assess the effect of EVs on thrombosis and VSMC biology respectively. As the field of EVs is still in its origins, we were unable to identify and develop techniques to isolate PAI-1^+ PEV fraction from PEVs without inhibiting PAI-1 during the isolation process. Moreover, all bench-top experiments were performed using EVs isolated from healthy volunteers. We are limited by the current extraction techniques for the quantity of EVs isolated from each blood sample. Moreover, great heterogeneity exists between multiple blood draws in the same individual.

Finally, the effect of PAI-1^+ PEV on VSMC phenotypic switching is not testable in an *in vivo* study. To date, animal models do not exist where we can manipulate EV production or exogenously administer EVs without initiating the host inflammatory response. Therapeutic development of these observations will likely rely on PAI-1 inhibition – a strategy previously demonstrated to be efficacious in mouse models of arterial injury.[261, 262]

6.5 Future directions

This thesis highlights a novel role of PAI-1 and PAI-1^+ PEV as a biomarker for unplanned revascularization and major adverse cardiac events, respectively. As these findings were based on retrospective observational studies (despite using a prospective registry), future studies would benefit from prospective observational studies or biomarker studies where measured PAI-1 levels guide treatment decisions and intense clinical follow-up strategy for optimization of cardiovascular risk factors. Our clinical outcomes were limited to a one-year follow-up period, where we saw a trend between plasma PAI-1 levels and unplanned revascularization. Future projects will evaluate three and five-year outcomes in this population by chart review.

Moreover, our study portends a mechanistic insight of PAI-1^+ PEV in mediating the pro-inflammatory VSMC phenotype driving neointimal formation. However, these studies are currently limited to the *in vitro* setting and experiments performed using VSMC cell culture model. We propose a future *in vivo* study employing the New Zealand white rabbit models extensively studied in the Hibbert laboratory where we previously evaluated post-PCI vascular healing and intravascular imaging by optical coherence tomography (data not yet published). New Zealand white rabbits will be anesthesized and undergo balloon angioplasty of the abdominal aorta to induce endothelial damage followed by randomization to PCI receiving bare-metal stents (BMS) or drug-eluting stents (DES) in a 1:1 fashion. Moreover, within the BMS versus DMS, rabbits will be randomized to vehicle, tiplaxtinin, or TM5275 coated stents. Based on previous experience with this model, we anticipate a neointimal area of 0.75 ± 0.22 mm^2 at 6 weeks and would consider a 30% reduction in neointimal area to be a significant difference (we currently achieve about 50% reduction with modern drug eluting stents – which will be observed with the RCS). To achieve 80% power with type I error (α) = 0.05, we require 16 rabbits per group (Figure 6-2).

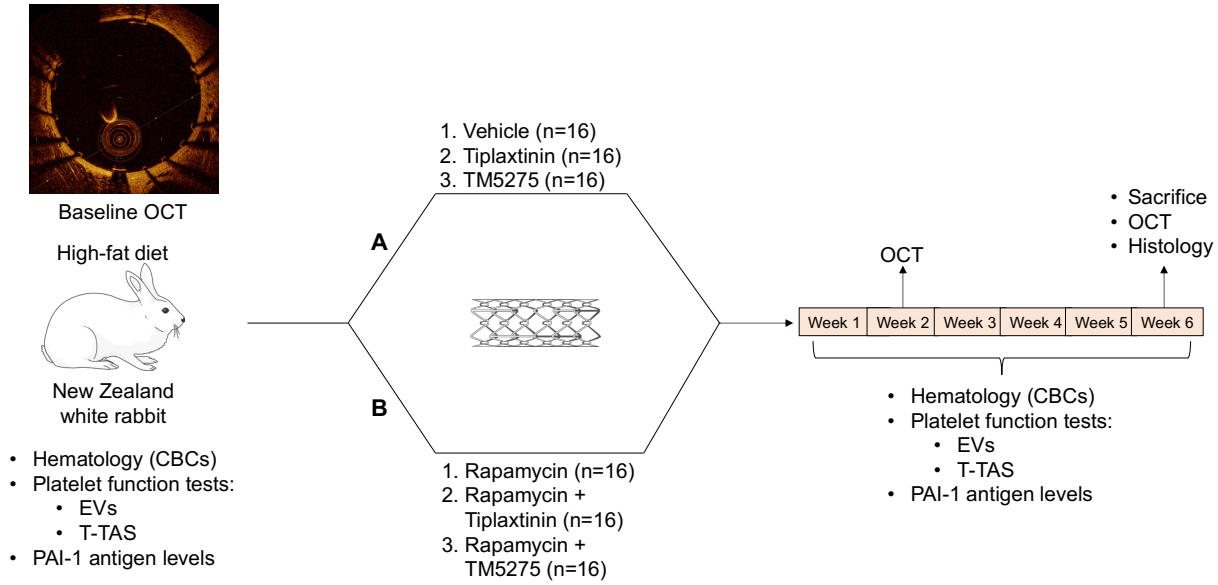


Figure 6-2. New Zealand white rabbit *in vivo* model to evaluate the role of PAI-1 PEV in neointimal formation.

Rabbits will be anesthetized and undergo balloon angioplasty to damage the endothelium followed by the randomization to stent implantation in the abdominal aorta of one of the following: (i) vehicle-coated stent (VCS), (ii) Rapamycin-coated (aka Sirolimus) stent (RCS) as a positive control, (iii) 10 $\boldsymbol{\mu}$M tiplaxtinin (PAI-039)-coated stent (TCS), or (iv) 0.1 uM TM5275.[80] All rabbits will receive contemporary medical therapy in the form of DAPT via rectal gel to minimize stent thrombosis throughout the study. Based on previous experience with our model, we anticipate a neointimal area of 0.75 ± 0.22 mm^2 at 6 weeks and would consider a 30% reduction in neointimal area to be a significant difference (we currently achieve about 50% reduction with modern drug eluting stents – which will be observed with the RCS). To achieve 80% power with type I error ($\boldsymbol{\alpha}$) = 0.05, we require 16 rabbits per group.

Future studies will allow the isolation of PAI-1^{+} PEV without inhibiting PAI-1 and allow the development of an *in vivo* animal model where EV production may be manipulated or allow multiple exogenous administration of EVs without propagating the inflammatory response.

Bibliography (or References)

1. Simard T, Hibbert B, Ramirez FD, Froeschl M, Chen YX, O'Brien ER. The evolution of coronary stents: a brief review. *Can J Cardiol*. Jan 2014;30(1):35-45. doi:10.1016/j.cjca.2013.09.012
2. Colman RW. Are hemostasis and thrombosis two sides of the same coin? *J Exp Med*. Mar 20 2006;203(3):493-5. doi:10.1084/jem.20060217
3. Watson T, Shantsila E, Lip GY. Mechanisms of thrombogenesis in atrial fibrillation: Virchow's triad revisited. *Lancet*. Jan 10 2009;373(9658):155-66. doi:10.1016/S0140-6736(09)60040-4
4. Broos K, Feys HB, De Meyer SF, Vanhoorelbeke K, Deckmyn H. Platelets at work in primary hemostasis. *Blood Rev*. Jul 2011;25(4):155-67. doi:10.1016/j.blre.2011.03.002
5. Bagoly Z, Koncz Z, Harsfalvi J, Muszbek L. Factor XIII, clot structure, thrombosis. *Thromb Res*. Mar 2012;129(3):382-7. doi:10.1016/j.thromres.2011.11.040
6. Chapin JC, Hajjar KA. Fibrinolysis and the control of blood coagulation. *Blood Rev*. Jan 2015;29(1):17-24. doi:10.1016/j.blre.2014.09.003
7. Hoylaerts M, Rijken DC, Lijnen HR, Collen D. Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of fibrin. *J Biol Chem*. Mar 25 1982;257(6):2912-9.
8. van Meijer M, Pannekoek, H. Structure of Plasminogen Activator Inhibitor 1 (PAI-1) and its Function in Fibrinolysis: an Update. *Fibrinolysis*. September 1995 1995;9(5):263-276. doi:10.1016/S0268-9499(95)80015-8
9. De Taeye B, Smith LH, Vaughan DE. Plasminogen activator inhibitor-1: a common denominator in obesity, diabetes and cardiovascular disease. *Curr Opin Pharmacol*. Apr 2005;5(2):149-154. doi:10.1016/j.coph.2005.01.007
10. Meade TW, Ruddock V, Stirling Y, Chakrabarti R, Miller GJ. Fibrinolytic activity, clotting factors, and long-term incidence of ischaemic heart disease in the Northwick Park Heart Study. *Lancet*. Oct 30 1993;342(8879):1076-9. doi:10.1016/0140-6736(93)92062-X
11. Binder BR, Christ G, Gruber F, et al. Plasminogen activator inhibitor 1: physiological and pathophysiological roles. *News Physiol Sci*. Apr 2002;17:56-61. doi:10.1152/nips.01369.2001
12. Carrell RW, Pemberton PA, Boswell DR. The serpins: evolution and adaptation in a family of protease inhibitors. *Cold Spring Harb Symp Quant Biol*. 1987;52:527-35. doi:10.1101/SQB.1987.052.01.060
13. Vaughan DE. PAI-1 and atherothrombosis. *J Thromb Haemost*. Aug 2005;3(8):1879-83. doi:10.1111/j.1538-7836.2005.01420.x
14. Strandberg L, Lawrence D, Ny T. The organization of the human-plasminogen-activator-inhibitor-1 gene. Implications on the evolution of the serine-protease inhibitor family. *Eur J Biochem*. Oct 1 1988;176(3):609-16. doi:10.1111/j.1432-1033.1988.tb14320.x
15. Loskutoff DJ. Regulation of PAI-1 Gene Expression. *Fibrinolysis*. 1991;5:197-206. doi:10.1016/0268-9499(91)90001-K
16. Loskutoff DJ, Linders M, Keijer J, Veerman H, van Heerikhuizen H, Pannekoek H. Structure of the human plasminogen activator inhibitor 1 gene: nonrandom distribution of introns. *Biochemistry*. Jun 30 1987;26(13):3763-8. doi:10.1021/bi00387a004
17. Brown NJ, Kim K-S, Chen Y-Q, et al. Synergistic Effect of Adrenal Steroids and Angiotensin II on Plasminogen Activator Inhibitor-1 Production. *The Journal of Clinical Endocrinology & Metabolism*. 2000;85(1):336-344. doi:10.1210/jcem.85.1.6305
18. van Zonneveld AJ, Curriden SA, Loskutoff DJ. Type 1 plasminogen activator inhibitor gene: functional analysis and glucocorticoid regulation of its promoter. *Proc Natl Acad Sci U S A*. Aug 1988;85(15):5525-9.
19. Skurk T, Lee YM, Hauner H. Angiotensin II and its metabolites stimulate PAI-1 protein release from human adipocytes in primary culture. *Hypertension*. May 2001;37(5):1336-40.

20. Brown NJ, Kim KS, Chen YQ, et al. Synergistic effect of adrenal steroids and angiotensin II on plasminogen activator inhibitor-1 production. *J Clin Endocrinol Metab*. Jan 2000;85(1):336-44. doi:10.1210/jcem.85.1.6305
21. Eriksson P, Nilsson L, Karpe F, Hamsten A. Very-low-density lipoprotein response element in the promoter region of the human plasminogen activator inhibitor-1 gene implicated in the impaired fibrinolysis of hypertriglyceridemia. *Arterioscler Thromb Vasc Biol*. Jan 1998;18(1):20-6. doi:10.1161/01.ATV.18.1.20
22. Verges B. Pathophysiology of diabetic dyslipidaemia: where are we? *Diabetologia*. May 2015;58(5):886-99. doi:10.1007/s00125-015-3525-8
23. Pont F, Duvillard L, Florentin E, Gambert P, Verges B. Early kinetic abnormalities of apoB-containing lipoproteins in insulin-resistant women with abdominal obesity. *Arterioscler Thromb Vasc Biol*. Oct 1 2002;22(10):1726-32.
24. Chen Y-Q, Su M, Walia RR, Hao Q, Covington JW, Vaughan DE. Sp1 Sites Mediate Activation of the Plasminogen Activator Inhibitor-1 Promoter by Glucose in Vascular Smooth Muscle Cells. *Journal of Biological Chemistry*. April 3, 1998 1998;273(14):8225-8231. doi:10.1074/jbc.273.14.8225
25. Song C, Burgess S, Eicher JD, O'Donnell CJ, Johnson AD. Causal Effect of Plasminogen Activator Inhibitor Type 1 on Coronary Heart Disease. *J Am Heart Assoc*. May 26 2017;6(6)doi:10.1161/JAHA.116.004918
26. Dennler S, Itoh S, Vivien D, ten Dijke P, Huet S, Gauthier JM. Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *EMBO J*. Jun 01 1998;17(11):3091-100. doi:10.1093/emboj/17.11.3091
27. Kunz C, Pebler S, Otte J, von der Ahe D. Differential regulation of plasminogen activator and inhibitor gene transcription by the tumor suppressor p53. *Nucleic Acids Res*. Sep 25 1995;23(18):3710-7.
28. Boekholdt SM, Bijsterveld NR, Moons AH, Levi M, Buller HR, Peters RJ. Genetic variation in coagulation and fibrinolytic proteins and their relation with acute myocardial infarction: a systematic review. *Circulation*. Dec 18 2001;104(25):3063-8. doi:10.1161/hc5001.100793
29. Huang J, Sabater-Lleal M, Asselbergs FW, et al. Genome-wide association study for circulating levels of PAI-1 provides novel insights into its regulation. *Blood*. Dec 06 2012;120(24):4873-81. doi:10.1182/blood-2012-06-436188
30. Koch W, Schrempf M, Erl A, et al. 4G/5G polymorphism and haplotypes of SERPINE1 in atherosclerotic diseases of coronary arteries. *Thromb Haemost*. Jun 2010;103(6):1170-80. doi:10.1160/TH09-10-0702
31. Alessi MC, Poggi M, Juhan-Vague I. Plasminogen activator inhibitor-1, adipose tissue and insulin resistance. *Curr Opin Lipidol*. Jun 2007;18(3):240-5. doi:10.1097/MOL.0b013e32814e6d29
32. Alessi MC, Juhan-Vague I. PAI-1 and the metabolic syndrome: links, causes, and consequences. *Arterioscler Thromb Vasc Biol*. Oct 2006;26(10):2200-7. doi:10.1161/01.ATV.0000242905.41404.68
33. Landin K, Tengborn L, Smith U. Elevated fibrinogen and plasminogen activator inhibitor (PAI-1) in hypertension are related to metabolic risk factors for cardiovascular disease. *J Intern Med*. Apr 1990;227(4):273-8.
34. Song J, Yoon YM, Jung HJ, Hong SH, Park H, Kim JQ. Plasminogen activator inhibitor-1 4G/5G promoter polymorphism and coagulation factor VII Arg353-->Gln polymorphism in Korean patients with coronary artery disease. *J Korean Med Sci*. Apr 2000;15(2):146-52. doi:10.3346/jkms.2000.15.2.146
35. Juhan-Vague I, Roul C, Alessi MC, Ardissone JP, Heim M, Vague P. Increased plasminogen activator inhibitor activity in non insulin dependent diabetic patients--relationship with plasma insulin. *Thromb Haemost*. Jun 30 1989;61(3):370-3.
36. Scarabin PY, Aillaud MF, Amouyel P, et al. Associations of fibrinogen, factor VII and PAI-1 with baseline findings among 10,500 male participants in a prospective study of myocardial infarction--the PRIME Study. Prospective Epidemiological Study of Myocardial Infarction. *Thromb Haemost*. Nov 1998;80(5):749-56.

37. Tjarnlund-Wolf A, Brogren H, Lo EH, Wang X. Plasminogen activator inhibitor-1 and thrombotic cerebrovascular diseases. *Stroke*. Oct 2012;43(10):2833-9. doi:10.1161/STROKEAHA.111.622217
38. Nikolopoulos GK, Bagos PG, Tsangaris I, et al. The association between plasminogen activator inhibitor type 1 (PAI-1) levels, PAI-1 4G/5G polymorphism, and myocardial infarction: a Mendelian randomization meta-analysis. *Clin Chem Lab Med*. Jul 2014;52(7):937-50. doi:10.1515/cclm-2013-1124
39. Brogren H, Wallmark K, Deinum J, Karlsson L, Jern S. Platelets retain high levels of active plasminogen activator inhibitor 1. *PLoS One*. 2011;6(11):e26762. doi:10.1371/journal.pone.0026762
40. Ginsburg D, Zeheb, R., Yang, A.Y., Rafferty, U.M., Andreasen, P.A., Nielsen, L., Dano, K., Lebo, R.V., Gelehrter, T.D. cDNA cloning of human plasminogen activator-inhibitor from endothelial cells. *Journal of Clinical Investigation*. 1986;78(6):1673-1680. doi:10.1172/JCI112761
41. van Mourik JA, Lawrence DA, Loskutoff DJ. Purification of an inhibitor of plasminogen activator (antiactivator) synthesized by endothelial cells. *J Biol Chem*. Dec 10 1984;259(23):14914-21.
42. Jensen JK, Thompson LC, Bucci JC, et al. Crystal structure of plasminogen activator inhibitor-1 in an active conformation with normal thermodynamic stability. *J Biol Chem*. Aug 26 2011;286(34):29709-17. doi:10.1074/jbc.M111.236554
43. Declerck PJ, Gils A, De Taeye B. Use of mouse models to study plasminogen activator inhibitor-1. *Methods Enzymol*. 2011;499:77-104. doi:10.1016/B978-0-12-386471-0.00005-5
44. Sherman PM, Lawrence DA, Yang AY, et al. Saturation mutagenesis of the plasminogen activator inhibitor-1 reactive center. *J Biol Chem*. Apr 15 1992;267(11):7588-95.
45. Lin Z, Jiang L, Yuan C, et al. Structural basis for recognition of urokinase-type plasminogen activator by plasminogen activator inhibitor-1. *J Biol Chem*. Mar 04 2011;286(9):7027-32. doi:10.1074/jbc.M110.204537
46. Dewilde M, Strelkov SV, Rabijns A, Declerck PJ. High quality structure of cleaved PAI-1-stab. *J Struct Biol*. Feb 2009;165(2):126-32. doi:10.1016/j.jsb.2008.11.001
47. Lawrence D, Strandberg L, Grundstrom T, Ny T. Purification of active human plasminogen activator inhibitor 1 from Escherichia coli. Comparison with natural and recombinant forms purified from eucaryotic cells. *Eur J Biochem*. Dec 22 1989;186(3):523-33. doi:10.1111/j.1432-1033.1989.tb15238.x
48. Yarmolinsky J, Bordin Barbieri N, Weinmann T, Ziegelmann PK, Duncan BB, Ines Schmidt M. Plasminogen activator inhibitor-1 and type 2 diabetes: a systematic review and meta-analysis of observational studies. *Sci Rep*. Jan 27 2016;6:17714. doi:10.1038/srep17714
49. Ghosh AK, Vaughan DE. PAI-1 in tissue fibrosis. *J Cell Physiol*. Feb 2012;227(2):493-507. doi:10.1002/jcp.22783
50. Levin EG, Santell L. Conversion of the active to latent plasminogen activator inhibitor from human endothelial cells. *Blood*. Oct 1987;70(4):1090-8.
51. Loskutoff DJ, Curriden SA. The fibrinolytic system of the vessel wall and its role in the control of thrombosis. *Ann N Y Acad Sci*. 1990;598:238-47.
52. Kruithof EK, Nicolosa G, Bachmann F. Plasminogen activator inhibitor 1: development of a radioimmunoassay and observations on its plasma concentration during venous occlusion and after platelet aggregation. *Blood*. Nov 1987;70(5):1645-53.
53. Booth NA, Robbie LA, Croll AM, Bennett B. Lysis of platelet-rich thrombi: the role of PAI-1. *Ann N Y Acad Sci*. Dec 4 1992;667:70-80. doi:10.1111/j.1749-6632.1992.tb51599.x
54. Sakata Y, Murakami T, Noro A, Mori K, Matsuda M. The specific activity of plasminogen activator inhibitor-1 in disseminated intravascular coagulation with acute promyelocytic leukemia. *Blood*. May 01 1991;77(9):1949-57.
55. Gils A, Declerck PJ. Plasminogen activator inhibitor-1. *Curr Med Chem*. Sep 2004;11(17):2323-34.
56. Sancho E, Tonge DW, Hockney RC, Booth NA. Purification and characterization of active and stable recombinant plasminogen-activator inhibitor accumulated at high levels in Escherichia coli. *Eur J Biochem*. Aug 15 1994;224(1):125-34.

57. Jensen JK, Wind T, Andreasen PA. The vitronectin binding area of plasminogen activator inhibitor-1, mapped by mutagenesis and protection against an inactivating organochemical ligand. *FEBS Lett*. Jun 19 2002;521(1-3):91-4. doi:10.1016/S0014-5793(02)02830-2
58. Xu Z, Balsara RD, Gorlatova NV, Lawrence DA, Castellino FJ, Ploplis VA. Conservation of critical functional domains in murine plasminogen activator inhibitor-1. *J Biol Chem*. Apr 23 2004;279(17):17914-20. doi:10.1074/jbc.M314197200
59. Zhou A, Huntington JA, Pannu NS, Carrell RW, Read RJ. How vitronectin binds PAI-1 to modulate fibrinolysis and cell migration. *Nat Struct Biol*. Jul 2003;10(7):541-4. doi:10.1038/nsb943
60. Seiffert D. Constitutive and regulated expression of vitronectin. *Histol Histopathol*. Jul 1997;12(3):787-97.
61. Luo M, Ji Y, Luo Y, Li R, Fay WP, Wu J. Plasminogen Activator Inhibitor-1 Regulates the Vascular Expression of Vitronectin. *J Thromb Haemost*. Oct 13 2017;doi:10.1111/jth.13869
62. Sudhof TC, Rothman JE. Membrane fusion: grappling with SNARE and SM proteins. *Science*. Jan 23 2009;323(5913):474-7. doi:10.1126/science.1161748
63. Blair P, Flaumenhaft R. Platelet alpha-granules: basic biology and clinical correlates. *Blood Rev*. Jul 2009;23(4):177-89. doi:10.1016/j.blre.2009.04.001
64. Robbie LA, Bennett B, Croll AM, Brown PA, Booth NA. Proteins of the fibrinolytic system in human thrombi. *Thromb Haemost*. Jan 1996;75(1):127-33.
65. Fay WP, Murphy JG, Owen WG. High concentrations of active plasminogen activator inhibitor-1 in porcine coronary artery thrombi. *Arterioscler Thromb Vasc Biol*. Oct 1996;16(10):1277-84. doi:10.1161/01.ATV.16.10.1277
66. Fay WP, Eitzman DT, Shapiro AD, Madison EL, Ginsburg D. Platelets inhibit fibrinolysis in vitro by both plasminogen activator inhibitor-1-dependent and -independent mechanisms. *Blood*. Jan 15 1994;83(2):351-6.
67. Roth GJ, Hickey MJ, Chung DW, Hickstein DD. Circulating human blood platelets retain appreciable amounts of poly (A)+ RNA. *Biochem Biophys Res Commun*. Apr 28 1989;160(2):705-10. doi:10.1016/0006-291X(89)92490-X
68. Brogren H, Karlsson L, Andersson M, Wang L, Erlinge D, Jern S. Platelets synthesize large amounts of active plasminogen activator inhibitor 1. *Blood*. Dec 15 2004;104(13):3943-8. doi:10.1182/blood-2004-04-1439
69. Landry P, Plante I, Ouellet DL, Perron MP, Rousseau G, Provost P. Existence of a microRNA pathway in anucleate platelets. *Nat Struct Mol Biol*. Sep 2009;16(9):961-6. doi:10.1038/nsmb.1651
70. Corduan A, Ple H, Laffont B, et al. Dissociation of SERPINE1 mRNA from the translational repressor proteins Ago2 and TIA-1 upon platelet activation. *Thromb Haemost*. May 2015;113(5):1046-59. doi:10.1160/TH14-07-0622
71. Weyrich AS, Lindemann S, Tolley ND, et al. Change in protein phenotype without a nucleus: translational control in platelets. *Semin Thromb Hemost*. Aug 2004;30(4):491-8. doi:10.1055/s-2004-833484
72. Kawai T, Lal A, Yang X, Galban S, Mazan-Mamczarz K, Gorospe M. Translational control of cytochrome c by RNA-binding proteins TIA-1 and HuR. *Mol Cell Biol*. Apr 2006;26(8):3295-307. doi:10.1128/MCB.26.8.3295-3307.2006
73. Newby AC, Zaltsman AB. Molecular mechanisms in intimal hyperplasia. *J Pathol*. Feb 2000;190(3):300-9. doi:10.1002/(SICI)1096-9896(200002)190:3<300::AID-PATH596>3.0.CO;2-I
74. Ji Y, Weng Z, Fish P, et al. Pharmacological Targeting of Plasminogen Activator Inhibitor-1 Decreases Vascular Smooth Muscle Cell Migration and Neointima Formation. *Arterioscler Thromb Vasc Biol*. Nov 2016;36(11):2167-2175. doi:10.1161/ATVBAHA.116.308344
75. Czekay RP, Wilkins-Port CE, Higgins SP, et al. PAI-1: An Integrator of Cell Signaling and Migration. *Int J Cell Biol*. 2011;2011:562481. doi:10.1155/2011/562481

76. Degryse B, Neels JG, Czekay RP, Aertgeerts K, Kamikubo Y, Loskutoff DJ. The low density lipoprotein receptor-related protein is a motogenic receptor for plasminogen activator inhibitor-1. *J Biol Chem*. May 21 2004;279(21):22595-604. doi:10.1074/jbc.M313004200
77. Kozlova N, Jensen JK, Chi TF, Samoylenko A, Kietzmann T. PAI-1 modulates cell migration in a LRP1-dependent manner via beta-catenin and ERK1/2. *Thromb Haemost*. May 2015;113(5):988-98. doi:10.1160/TH14-08-0678
78. Mimuro J, Loskutoff DJ. Purification of a protein from bovine plasma that binds to type 1 plasminogen activator inhibitor and prevents its interaction with extracellular matrix. Evidence that the protein is vitronectin. *J Biol Chem*. Jan 15 1989;264(2):936-9.
79. Thogersen AM, Jansson JH, Boman K, et al. High plasminogen activator inhibitor and tissue plasminogen activator levels in plasma precede a first acute myocardial infarction in both men and women: evidence for the fibrinolytic system as an independent primary risk factor. *Circulation*. Nov 24 1998;98(21):2241-7. doi:10.1161/01.cir.98.21.2241
80. Johansson L, Jansson JH, Boman K, Nilsson TK, Stegmayr B, Hallmans G. Tissue plasminogen activator, plasminogen activator inhibitor-1, and tissue plasminogen activator/plasminogen activator inhibitor-1 complex as risk factors for the development of a first stroke. *Stroke*. Jan 2000;31(1):26-32. doi:10.1161/01.STR.31.1.26
81. Meigs JB, O'Donnell C J, Tofler GH, et al. Hemostatic markers of endothelial dysfunction and risk of incident type 2 diabetes: the Framingham Offspring Study. *Diabetes*. Feb 2006;55(2):530-7. doi:10.2337/diabetes.55.02.06.db05-1041
82. Trost S, Pratley RE, Sobel BE. Impaired fibrinolysis and risk for cardiovascular disease in the metabolic syndrome and type 2 diabetes. journal article. *Current Diabetes Reports*. January 01 2006;6(1):47-54. doi:10.1007/s11892-006-0052-5
83. Sobel BE, Taatjes DJ, Schneider DJ. Intramural plasminogen activator inhibitor type-1 and coronary atherosclerosis. *Arterioscler Thromb Vasc Biol*. Nov 01 2003;23(11):1979-89. doi:10.1161/01.ATV.0000091250.53231.4D
84. Brazionis L, Rowley K, Jenkins A, Itsiopoulos C, O'Dea K. Plasminogen activator inhibitor-1 activity in type 2 diabetes: a different relationship with coronary heart disease and diabetic retinopathy. *Arterioscler Thromb Vasc Biol*. Apr 2008;28(4):786-91. doi:10.1161/ATVBAHA.107.160168
85. Scheer FA, Shea SA. Human circadian system causes a morning peak in prothrombotic plasminogen activator inhibitor-1 (PAI-1) independent of the sleep/wake cycle. *Blood*. Jan 23 2014;123(4):590-3. doi:10.1182/blood-2013-07-517060
86. Simpson AJ, Gray RS, Moore NR, Booth NA. The effects of chronic smoking on the fibrinolytic potential of plasma and platelets. *Br J Haematol*. Apr 1997;97(1):208-13.
87. Cooper JA, Nagelkirk PR, Coughlin AM, Pivarnik JM, Womack CJ. Temporal changes in tPA and PAI-1 after maximal exercise. *Med Sci Sports Exerc*. Nov 2004;36(11):1884-7.
88. Byrne CD, Wareham NJ, Martensz ND, Humphries SE, Metcalfe JC, Grainger DJ. Increased PAI activity and PAI-1 antigen occurring with an oral fat load: associations with PAI-1 genotype and plasma active TGF-beta levels. *Atherosclerosis*. Sep 1998;140(1):45-53.
89. Declerck PJ, Alessi MC, Verstreken M, Kruithof EK, Juhan-Vague I, Collen D. Measurement of plasminogen activator inhibitor 1 in biologic fluids with a murine monoclonal antibody-based enzyme-linked immunosorbent assay. *Blood*. Jan 1988;71(1):220-5.
90. Gils A, Declerck PJ. Modulation of plasminogen activator inhibitor 1 by Triton X-100--identification of two consecutive conformational transitions. *Thromb Haemost*. Aug 1998;80(2):286-91.
91. Andreasen PA, Egelund R, Jensen S, Rodenburg KW. Solvent effects on activity and conformation of plasminogen activator inhibitor-1. *Thromb Haemost*. Mar 1999;81(3):407-14.
92. Hekman CM, Loskutoff DJ. Endothelial cells produce a latent inhibitor of plasminogen activators that can be activated by denaturants. *J Biol Chem*. Sep 25 1985;260(21):11581-7.

93. Pieters M, Barnard SA, Loots DT, Rijken DC. The effects of residual platelets in plasma on plasminogen activator inhibitor-1 and plasminogen activator inhibitor-1-related assays. *PLoS One*. 2017;12(2):e0171271. doi:10.1371/journal.pone.0171271
94. Sidelmann J. The influence of centrifugation load on platelet number and PAI-1 antigen concentration in human plasma. *Fibrinolysis*. 1994/01/01/ 1994;8(Supplement 2):148-149. doi:https://doi.org/10.1016/0268-9499(94)90279-8
95. Vaughan DE. PAI-1 antagonists: the promise and the peril. *Trans Am Clin Climatol Assoc*. 2011;122:312-25.
96. Juhan-Vague I, Alessi MC. PAI-1, obesity, insulin resistance and risk of cardiovascular events. *Thromb Haemost*. Jul 1997;78(1):656-60.
97. Bastelica D, Morange P, Berthet B, et al. Stromal cells are the main plasminogen activator inhibitor-1-producing cells in human fat: evidence of differences between visceral and subcutaneous deposits. *Arterioscler Thromb Vasc Biol*. Jan 2002;22(1):173-8. doi:10.1161/hq0102.101552
98. Schafer K, Fujisawa K, Konstantinides S, Loskutoff DJ. Disruption of the plasminogen activator inhibitor 1 gene reduces the adiposity and improves the metabolic profile of genetically obese and diabetic ob/ob mice. *FASEB J*. Aug 2001;15(10):1840-2. doi:10.1096/fj.00-0750fje
99. Ma LJ, Mao SL, Taylor KL, et al. Prevention of obesity and insulin resistance in mice lacking plasminogen activator inhibitor 1. *Diabetes*. Feb 2004;53(2):336-46. doi:10.2337/diabetes.53.2.336
100. D'Agostino RB, Jr., Hamman RF, Karter AJ, et al. Cardiovascular disease risk factors predict the development of type 2 diabetes: the insulin resistance atherosclerosis study. *Diabetes Care*. Sep 2004;27(9):2234-40. doi:10.2337/diacare.27.9.2234
101. Nordt TK, Schneider DJ, Sobel BE. Augmentation of the synthesis of plasminogen activator inhibitor type-1 by precursors of insulin. A potential risk factor for vascular disease. *Circulation*. Jan 1994;89(1):321-30. doi:10.1161/01.CIR.89.1.321
102. Eitzman DT, Westrick RJ, Xu Z, Tyson J, Ginsburg D. Plasminogen activator inhibitor-1 deficiency protects against atherosclerosis progression in the mouse carotid artery. *Blood*. Dec 15 2000;96(13):4212-5.
103. Bini A, Fenoglio JJ, Jr., Mesa-Tejada R, Kudryk B, Kaplan KL. Identification and distribution of fibrinogen, fibrin, and fibrin(ogen) degradation products in atherosclerosis. Use of monoclonal antibodies. *Arteriosclerosis*. Jan-Feb 1989;9(1):109-21. doi:10.1161/01.ATV.9.1.109
104. Naito M, Funaki C, Hayashi T, et al. Substrate-bound fibrinogen, fibrin and other cell attachment-promoting proteins as a scaffold for cultured vascular smooth muscle cells. *Atherosclerosis*. Oct 1992;96(2-3):227-34. doi:10.1016/0021-9150(92)90069-S
105. Ploplis VA. Effects of altered plasminogen activator inhibitor-1 expression on cardiovascular disease. *Curr Drug Targets*. Nov 2011;12(12):1782-9. doi:10.2174/138945011797635803
106. Eren M, Painter CA, Atkinson JB, Declerck PJ, Vaughan DE. Age-dependent spontaneous coronary arterial thrombosis in transgenic mice that express a stable form of human plasminogen activator inhibitor-1. *Circulation*. Jul 23 2002;106(4):491-6. doi:10.1161/01.CIR.0000023186.60090.FB
107. Battes LC, Akkerhuis KM, Cheng JM, et al. Circulating acute phase proteins in relation to extent and composition of coronary atherosclerosis and cardiovascular outcome: Results from the ATHEROREMO-IVUS study. *Int J Cardiol*. 2014;177(3):847-853. doi:10.1016/j.ijcard.2014.11.001
108. ER OB, Ma X, Simard T, Pourdjabbar A, Hibbert B. Pathogenesis of neointima formation following vascular injury. *Cardiovasc Hematol Disord Drug Targets*. Mar 1 2011;11(1):30-9. doi:10.2174/187152911795945169
109. Pourdjabbar A, Hibbert B, Simard T, Ma X, R. O' Brien E. Pathogenesis of Neointima Formation Following Vascular Injury. *Cardiovascular & Haematological Disorders - Drug Targets(Formerly Current Drug Targets - Cardiovascular & Hematological Disorders)*. // 2011;11(1):30-39. doi:10.2174/187152911795945169

110. Cassese S, Byrne RA, Tada T, et al. Incidence and predictors of restenosis after coronary stenting in 10 004 patients with surveillance angiography. *Heart*. Jan 2014;100(2):153-9. doi:10.1136/heartjnl-2013-304933
111. Magalhaes MA, Minha S, Chen F, et al. Clinical presentation and outcomes of coronary in-stent restenosis across 3-stent generations. *Circ Cardiovasc Interv*. Dec 2014;7(6):768-76. doi:10.1161/circinterventions.114.001341
112. Katsaros KM, Speidl WS, Kastl SP, et al. Plasminogen activator inhibitor-1 predicts coronary in-stent restenosis of drug-eluting stents. *J Thromb Haemost*. 2008;6(3):508-513. doi:10.1111/j.1538-7836.2007.02884.x
113. Byrne RA, Joner M, Kastrati A. Stent thrombosis and restenosis: what have we learned and where are we going? The Andreas Gruntzig Lecture ESC 2014. *Eur Heart J*. Dec 14 2015;36(47):3320-31. doi:10.1093/eurheartj/ehv511
114. Ellis SG, Kereiakes DJ, Metzger DC, et al. Everolimus-Eluting Bioresorbable Scaffolds for Coronary Artery Disease. *N Engl J Med*. Nov 12 2015;373(20):1905-15. doi:10.1056/NEJMoa1509038
115. Nguyen KT, Shaikh N, Shukla KP, Su SH, Eberhart RC, Tang L. Molecular responses of vascular smooth muscle cells and phagocytes to curcumin-eluting bioresorbable stent materials. *Biomaterials*. 10/2004 2004;25(23):5333-5346. Not in File.
116. Kandzari DE, Leon MB, Popma JJ, et al. Comparison of Zotarolimus-Eluting and Sirolimus-Eluting Stents in Patients With Native Coronary Artery Disease. A Randomized Controlled Trial. *Journal of the American College of Cardiology*. 2006 2006;48(12):2440-2447. Not in File.
117. Di Santo P, Simard T, Ramirez FD, et al. Does stent strut design impact clinical outcomes: comparative safety and efficacy of Endeavor Resolute versus Resolute Integrity zotarolimus-eluting stents. *Clin Invest Med*. Oct 07 2015;38(5):E296-304.
118. Giles K, Hibbert B, Drzymala L, et al. Three year clinical outcomes with zotarolimus (Endeavor) drug-eluting stents in an unrestricted contemporary practice. *Int J Cardiol*. Sep 20 2013;168(1):595-6. doi:10.1016/j.ijcard.2013.01.234
119. Schafer K, Schroeter MR, Dellas C, et al. Plasminogen activator inhibitor-1 from bone marrow-derived cells suppresses neointimal formation after vascular injury in mice. *Arterioscler Thromb Vasc Biol*. Jun 2006;26(6):1254-9. doi:10.1161/01.ATV.0000215982.14003.b7
120. Strauss BH, Lau HK, Bowman KA, et al. Plasma urokinase antigen and plasminogen activator inhibitor-1 antigen levels predict angiographic coronary restenosis. *Circulation*. Oct 12 1999;100(15):1616-22. doi:10.1161/01.CIR.100.15.1616
121. Prisco D, Fedi S, Antonucci E, et al. Postprocedural PAI-1 activity is a risk marker of subsequent clinical restenosis in patients both with and without stent implantation after elective balloon PTCA. *Thromb Res*. Nov 01 2001;104(3):181-6. doi:10.1016/S0049-3848(01)00360-7
122. Leon MB, Baim DS, Popma JJ, et al. A clinical trial comparing three antithrombotic-drug regimens after coronary-artery stenting. Stent Anticoagulation Restenosis Study Investigators. *N Engl J Med*. Dec 03 1998;339(23):1665-71. doi:10.1056/NEJM199812033392303
123. Schömig A, Neumann F-J, Kastrati A, et al. A Randomized Comparison of Antiplatelet and Anticoagulant Therapy after the Placement of Coronary-Artery Stents. *New England Journal of Medicine*. 1996;334(17):1084-1089. doi:10.1056/nejm199604253341702
124. Muldowney JA, 3rd, Stringham JR, Levy SE, et al. Antiproliferative agents alter vascular plasminogen activator inhibitor-1 expression: a potential prothrombotic mechanism of drug-eluting stents. *Arterioscler Thromb Vasc Biol*. Feb 2007;27(2):400-6. doi:10.1161/01.ATV.0000254677.12861.b8
125. Biemond BJ, Levi M, Coronel R, Janse MJ, ten Cate JW, Pannekoek H. Thrombolysis and reocclusion in experimental jugular vein and coronary artery thrombosis. Effects of a plasminogen activator inhibitor type 1-neutralizing monoclonal antibody. *Circulation*. Feb 15 1995;91(4):1175-81. doi:10.1161/01.CIR.91.4.1175

126. Elokdah H, Abou-Gharbia M, Hennan JK, et al. Tiplaxtinin, a novel, orally efficacious inhibitor of plasminogen activator inhibitor-1: design, synthesis, and preclinical characterization. *J Med Chem*. Jul 01 2004;47(14):3491-4. doi:10.1021/jm049766q
127. Pautus S, Alami M, Adam F, et al. Characterization of the Annonaceous acetogenin, annonacinone, a natural product inhibitor of plasminogen activator inhibitor-1. *Sci Rep*. Nov 23 2016;6:36462. doi:10.1038/srep36462
128. Fortenberry YM. Plasminogen activator inhibitor-1 inhibitors: a patent review (2006-present). *Expert Opin Ther Pat*. Jul 2013;23(7):801-15. doi:10.1517/13543776.2013.782393
129. Simone TM, Higgins SP, Higgins CE, Lennartz MR, Higgins PJ. Chemical Antagonists of Plasminogen Activator Inhibitor-1: Mechanisms of Action and Therapeutic Potential in Vascular Disease. *J Mol Genet Med*. Oct 2014;8(3)doi:10.4172/1747-0862.1000125
130. Hennan JK, Elokdah H, Leal M, et al. Evaluation of PAI-039 [{1-benzyl-5-[4-(trifluoromethoxy)phenyl]-1H-indol-3-yl}(oxo)acetic acid], a novel plasminogen activator inhibitor-1 inhibitor, in a canine model of coronary artery thrombosis. *J Pharmacol Exp Ther*. Aug 2005;314(2):710-6. doi:10.1124/jpet.105.084129
131. Gorlatova NV, Cale JM, Elokdah H, et al. Mechanism of inactivation of plasminogen activator inhibitor-1 by a small molecule inhibitor. *J Biol Chem*. Mar 23 2007;282(12):9288-96. doi:10.1074/jbc.M611642200
132. Hennan JK, Morgan GA, Swillo RE, et al. Effect of tiplaxtinin (PAI-039), an orally bioavailable PAI-1 antagonist, in a rat model of thrombosis. *J Thromb Haemost*. Sep 2008;6(9):1558-64. doi:10.1111/j.1538-7836.2008.03063.x
133. Lucore CL, Sobel BE. Interactions of tissue-type plasminogen activator with plasma inhibitors and their pharmacologic implications. *Circulation*. Mar 1988;77(3):660-9.
134. Srikanthan K, Feyh A, Visweshwar H, Shapiro JI, Sodhi K. Systematic Review of Metabolic Syndrome Biomarkers: A Panel for Early Detection, Management, and Risk Stratification in the West Virginian Population. *Int J Med Sci*. 2016;13(1):25-38. doi:10.7150/ijms.13800
135. Thogersen AM, Jansson JH, Boman K, et al. High plasminogen activator inhibitor and tissue plasminogen activator levels in plasma precede a first acute myocardial infarction in both men and women - Evidence for the fibrinolytic system as an independent primary risk factor. *Circulation*. 1998;98(21):2241-2247.
136. Trost S, Pratley R, Sobel B. Impaired fibrinolysis and risk for cardiovascular disease in the metabolic syndrome and type 2 diabetes. *Curr Diab Rep*. Feb 2006;6(1):47-54.
137. Brazionis L, Rowley K, Jenkins A, Itsiopoulos C, O'Dea K. Plasminogen activator inhibitor-1 activity in type 2 diabetes - A different relationship with coronary heart disease and diabetic retinopathy. *Arterioscler Thromb Vasc Biol*. 2008;28(4):786-791. doi:10.1161/ATVBAHA.107.160168
138. Anderson L, Oldridge N, Thompson DR, et al. Exercise-Based Cardiac Rehabilitation for Coronary Heart Disease: Cochrane Systematic Review and Meta-Analysis. *J Am Coll Cardiol*. Jan 05 2016;67(1):1-12. doi:10.1016/j.jacc.2015.10.044
139. Wells G, Shea, B., O'Connell, D., Welch, V., Losos, M., Tugwell, P. The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomised studies in meta-analyses. http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp
140. Wan X, Wang W, Liu J, Tong T. Estimating the sample mean and standard deviation from the sample size, median, range and/or interquartile range. *BMC Med Res Methodol*. Dec 19 2014;14:135. doi:10.1186/1471-2288-14-135
141. Sane DC, Stump DC, Topol EJ, et al. Correlation between baseline plasminogen activator inhibitor levels and clinical outcome during therapy with tissue plasminogen activator for acute myocardial infarction. *Thrombosis and haemostasis*. 1991;65(3):275-9.
142. Cortellaro M, Cofrancesco E, Boschetti C, et al. Increased Fibrin Turnover and High Pai-1 Activity as Predictors of Ischemic Events in Atherosclerotic Patients - a Case-Control Study. *Arterioscler Thromb*. 1993;13(10):1412-1417.

143. Brannstrom M, Jansson JH, Boman K, Nilsson TK. Endothelial Hemostatic Factors May Be Associated with Mortality in Patients on Long-Term Anticoagulant Treatment. *Thromb Haemost*. 1995;74(2):612-615.
144. JuhanVague I, Pyke SDM, Alessi MC, Jespersen J, Haverkate F, Thompson SG. Fibrinolytic factors and the risk of myocardial infarction or sudden death in patients with angina pectoris. *Circulation*. 1996;94(9):2057-2063.
145. Nordt TK, Moser M, Kohler B, et al. Augmented platelet aggregation as predictor of reocclusion after thrombolysis in acute myocardial infarction. *Thromb Haemost*. 1998;80(6):881-886.
146. Alaigh P, Hoffman CJ, Korlipara G, et al. Lipoprotein(a) level does not predict restenosis after percutaneous transluminal coronary angioplasty. *Arterioscler Thromb Vasc Biol*. 1998;18(8):1281-1286.
147. Moss AJ, Goldstein RE, Marder VJ, et al. Thrombogenic factors and recurrent coronary events. *Circulation*. May 18 1999;99(19):2517-22. doi:10.1161/01.cir.99.19.2517
148. Redondo M, Carroll VA, Mauron T, et al. Hemostatic and fibrinolytic parameters in survivors of myocardial infarction: A low plasma level of plasmin-alpha2-antiplasmin complex is an independent predictor of coronary re-events. *Blood Coagulation and Fibrinolysis*. 2001;12(1):17-24.
149. Fornitz GG, Nielsen P, Amtorp O, et al. Impaired fibrinolysis determines the outcome of percutaneus transluminal coronary angioplasty (PTCA). *Eur J Clin Invest*. 2001;31(7):586-592. doi:10.1046/j.1365-2362.2001.00855.x
150. Bogaty P, Poirier P, Simard S, Boyer L, Solymoss S, Dagenais GR. Biological profiles in subjects with recurrent acute coronary events compared with subjects with long-standing stable angina. *Circulation*. 2001;103(25):3062-3068.
151. Ganti AK, Potti A, Yegnanarayan R. Plasma tissue plasminogen activator and plasminogen activator inhibitor-1 levels in acute myocardial infarction. *Pathophysiol Haemost Thromb*. 2002;32(2):80-84. doi:10.1159/000065080
152. Lip GYH, Blann AD, Farooqi IS, Zarifis J, Sagar G, Beevers DG. Sequential alterations in haemorheology, endothelial dysfunction, platelet activation and thrombogenesis in relation to prognosis following acute stroke: The West Birmingham Stroke Project. *Blood Coagul Fibrinolysis*. 2002;13(4):339-347. doi:10.1097/00001721-200206000-00010
153. Inoue T, Yaguchi I, Mizoguchi K, et al. Accelerated plasminogen activator inhibitor may prevent late restenosis after coronary stenting in acute myocardial infarction. *Clin Cardiol*. 2003;26(3):153-157. doi:10.1002/clc.4960260311
154. Christ G, Nikfardjam M, Huber-Beckmann R, et al. Predictive value of plasma plasminogen activator inhibitor-1 for coronary restenosis: dependence on stent implantation and antithrombotic medication. *J Thromb Haemost*. Feb 2005;3(2):233-9. doi:10.1111/j.1538-7836.2004.01062.x
155. El-Menyar AA, Altamimi OM, Gomaa MM, et al. Clinical and biochemical predictors affect the choice and the short-term outcomes of different thrombolytic agents in acute myocardial infarction. *Coronary artery disease*. 2006;17(5):431-7.
156. Robinson SD, Ludlam CA, Boon NA, Newby DE. Endothelial fibrinolytic capacity predicts future adverse cardiovascular events in patients with coronary heart disease. *Arterioscler Thromb Vasc Biol*. 2007;27(7):1651-1656. doi:10.1161/ATVBAHA.107.143248
157. Thogersen AM, Nilsson TK, Weinehall L, et al. Changes in plasma C-reactive protein and hemostatic factors prior to and after a first myocardial infarction with a median follow-up time of 8 years. *Blood coagulation & fibrinolysis : an international journal in haemostasis and thrombosis*. 2009;20(5):340-6.
158. Akkus MN, Polat G, Yurtdas M, et al. Admission Levels of C-Reactive Protein and Plasminogen Activator Inhibitor-1 in Patients With Acute Myocardial Infarction With and Without Cardiogenic Shock or Heart Failure on Admission. *Int Heart J*. 2009;50(1):33-45.
159. Arikan H, Koc M, Tuglular S, Ozener C, Akoglu E. Elevated Plasma Levels of PAI-1 Predict Cardiovascular Events and Cardiovascular Mortality in Prevalent Peritoneal Dialysis Patients. *Ren Fail*. 2009;31(6):438-445. doi:10.1080/08860220902963772

160. Pineda J, Marin F, Marco P, et al. Premature coronary artery disease in young (age < 45) subjects: Interactions of lipid profile, thrombophilic and haemostatic markers. *Int J Cardiol*. 2009;136(2):222-225. doi:10.1016/j.ijcard.2008.04.020
161. Wennberg P, Wensley F, Di Angelantonio E, et al. Haemostatic and inflammatory markers are independently associated with myocardial infarction in men and women. *Thromb Res*. Jan 2012;129(1):68-73. doi:10.1016/j.thromres.2011.05.015
162. Yano Y, Hoshide S, Shimada K, Kario K. The Impact of Cigarette Smoking on 24-Hour Blood Pressure, Inflammatory and Hemostatic Activity, and Cardiovascular Risk in Japanese Hypertensive Patients. *J Clin Hypertens*. 2013;15(4):234-240. doi:10.1111/jch.12047
163. Iacoviello L, Agnoli C, De Curtis A, et al. Type 1 plasminogen activator inhibitor as a common risk factor for cancer and ischaemic vascular disease: the EPICOR study. *BMJ Open*. 2013;3(11)doi:10.1136/bmjopen-2013-003725
164. Yano Y, Nakazato M, Toshinai K, et al. Circulating Des-acyl Ghrelin Improves Cardiovascular Risk Prediction in Older Hypertensive Patients. *Am J Hypertens*. 2014;27(5):727-733. doi:10.1093/ajh/hpt232
165. Knudsen A, Katzenstein TL, Benfield T, et al. Plasma plasminogen activator inhibitor-1 predicts myocardial infarction in HIV-1-infected individuals. *AIDS (London, England)*. 2014;28(8):1171-9.
166. Golukhova EZ, Grigorian MV, Ryabinina MN, Bulaeva NI, Fortmann S, Serebruany VL. Independent Predictors of Major Adverse Events following Coronary Stenting over 28 Months of Follow-Up. *Cardiology*. 2015;132(3):176-181. doi:10.1159/000435909
167. Shah PK, Amin J. Low high density lipoprotein level is associated with increased restenosis rate after coronary angioplasty. *Circulation*. 1992;85(4):1279-85.
168. Gray RP, Yudkin JS, Patterson DL. Enzymatic evidence of impaired reperfusion in diabetic patients after thrombolytic therapy for acute myocardial infarction: a role for plasminogen activator inhibitor? *British heart journal*. 1993;70(6):530-6.
169. Malmberg K, Bavenholm P, Hamsten A. Clinical and biochemical factors associated with prognosis after myocardial infarction at a young age. *Journal of the American College of Cardiology*. 1994;24(3):592-9.
170. Brack MJ, More RS, Pringle S, Gershlick AH. Absence of a Prothrombotic State in Restenotic Patients. *Coronary Artery Dis*. 1994;5(6):501-506.
171. Jansson JH, Nilsson TK, Johnson O. von Willebrand factor, tissue plasminogen activator, and dehydroepiandrosterone sulphate predict cardiovascular death in a 10 year follow up of survivors of acute myocardial infarction. *Heart (British Cardiac Society)*. 1998;80(4):334-7.
172. Sinkovic A. Prognostic role of plasminogen-activator-inhibitor-1 levels in treatment with streptokinase of patients with acute myocardial infarction. *Clin Cardiol*. 2000;23(7):486-489.
173. Wiman B, Andersson T, Hallqvist J, Reuterwall C, Ahlbom A, deFaire U. Plasma levels of tissue plasminogen activator/plasminogen activator inhibitor-1 complex and von willebrand factor are significant risk markers for recurrent myocardial infarction in the Stockholm Heart Epidemiology Program (SHEEP) study. *Arterioscler Thromb Vasc Biol*. 2000;20(8):2019-2023.
174. Prisco D, Antonucci E, Fedi S, et al. D-Dimer increase after percutaneous transluminal angioplasty and clinical recurrence after primary revascularization in acute myocardial infarction? A pilot study. *Clin Exper Med*. 2001;1(4):219-224. doi:10.1007/s102380100006
175. Sargento L, Saldanha C, Monteiro J, Perdigao C, Martins e Silva J. Evidence of prolonged disturbances in the haemostatic, hemorheologic and inflammatory profiles in transmural myocardial infarction survivors. *Thrombosis and haemostasis*. 2003;89(5):892-903.
176. Marcucci R, Brogi D, Sofi F, et al. PAI-1 and homocysteine, but not lipoprotein (a) and thrombophilic polymorphisms, are independently associated with the occurrence of major adverse cardiac events after successful coronary stenting. *Heart*. Mar 2006;92(3):377-81. doi:10.1136/hrt.2005.061895

177. Schoebel FC, Peters AJ, Kreis I, Gradaus F, Heins M, Jax TW. Relevance of hemostasis on restenosis in clinically stable patients undergoing elective PTCA. *Thromb Res*. 2008;122(2):229-236. doi:10.1016/j.thromres.2007.10.007
178. Tofler GH, Massaro J, O'Donnell CJ, et al. Plasminogen activator inhibitor and the risk of cardiovascular disease: The Framingham Heart Study. *Thromb Res*. Apr 2016;140:30-5. doi:10.1016/j.thromres.2016.02.002
179. Garg N, Goyal N, Strawn TL, et al. Plasminogen Activator Inhibitor-1 and Vitronectin Expression Level and Stoichiometry Regulate Vascular Smooth Muscle Cell Migration through Physiological Collagen Matrices. *Journal of thrombosis and haemostasis : JTH*. 05/12 2010;8(8):1847-1854. doi:10.1111/j.1538-7836.2010.03907.x
180. Katsaros KM, Speidl WS, Kastl SP, et al. Plasminogen activator inhibitor-1 predicts coronary in-stent restenosis of drug-eluting stents. *J Thromb Haemost*. Mar 2008;6(3):508-13. doi:10.1111/j.1538-7836.2007.02884.x
181. Clowes AW, Reidy MA, Clowes MM. Kinetics of cellular proliferation after arterial injury. I. Smooth muscle growth in the absence of endothelium. *Lab Invest*. Sep 1983;49(3):327-33.
182. Iacoviello L, Agnoli C, De Curtis A, et al. Type 1 plasminogen activator inhibitor (PAI-1) and risk of acute coronary syndrome in the european prospective investigation into cancer (EPIC)-Italy cohort. *Blood Transfusion*. 2012;10:s37.
183. Angleton P, Chandler WL, Schmer G. Diurnal variation of tissue-type plasminogen activator and its rapid inhibitor (PAI-1). *Circulation*. 1989;79(1):101-106. doi:10.1161/01.cir.79.1.101
184. Chong NW, Codd V, Chan D, Samani NJ. Circadian clock genes cause activation of the human PAI-1 gene promoter with 4G/5G allelic preference. *FEBS Lett*. Aug 07 2006;580(18):4469-72. doi:10.1016/j.febslet.2006.07.014
185. Bae JM. A suggestion for quality assessment in systematic reviews of observational studies in nutritional epidemiology. *Epidemiol Health*. 2016;38:e2016014. doi:10.4178/epih.e2016014
186. Lau J, Ioannidis JP, Terrin N, Schmid CH, Olkin I. The case of the misleading funnel plot. *BMJ*. Sep 16 2006;333(7568):597-600. doi:10.1136/bmj.333.7568.597
187. Jung RG, Simard T, Labinaz A, et al. Role of plasminogen activator inhibitor-1 in coronary pathophysiology. *Thrombosis Research*. 2018/04/01/ 2018;164:54-62. doi:https://doi.org/10.1016/j.thromres.2018.02.135
188. Brogren H, Sihlbom C, Wallmark K, et al. Heterogeneous glycosylation patterns of human PAI-1 may reveal its cellular origin. *Thromb Res*. 2008;122(2):271-81. doi:10.1016/j.thromres.2008.04.008
189. Jung RG, Motazedian P, Ramirez FD, et al. Association between plasminogen activator inhibitor-1 and cardiovascular events: a systematic review and meta-analysis. *Thromb J*. 2018;16:12. doi:https://dx.doi.org/10.1186/s12959-018-0166-4
190. Fraser CG, Harris EK. Generation and application of data on biological variation in clinical chemistry. *Critical reviews in clinical laboratory sciences*. 1989;27(5):409-37. doi:10.3109/10408368909106595
191. Meijers WC, van der Velde AR, Muller Kobold AC, et al. Variability of biomarkers in patients with chronic heart failure and healthy controls. *Eur J Heart Fail*. Mar 2017;19(3):357-365. doi:10.1002/ejhf.669
192. Macy EM, Hayes TE, Tracy RP. Variability in the measurement of C-reactive protein in healthy subjects: implications for reference intervals and epidemiological applications. *Clinical Chemistry*. 1997;43(1):52-58.
193. Bay M, Kirk V, Parner J, et al. NT-proBNP: a new diagnostic screening tool to differentiate between patients with normal and reduced left ventricular systolic function. *Heart (British Cardiac Society)*. 2003;89(2):150-154.
194. Petersen PH, Fraser CG, Sandberg S, Goldschmidt H. The index of individuality is often a misinterpreted quantity characteristic. *Clin Chem Lab Med*. Jun 1999;37(6):655-61. doi:10.1515/CCLM.1999.102

195. Xu L, Jiang CQ, Lam TH, Bao B, Cheng KK, Thomas GN. Plasminogen activator inhibitor-1 and HbA1c defined prediabetes: the Guangzhou Biobank Cohort Study-CVD. *Clin Endocrinol (Oxf)*. Apr 2011;74(4):528-31. doi:10.1111/j.1365-2265.2010.03948.x
196. Kaur J. A comprehensive review on metabolic syndrome. *Cardiology research and practice*. 2014;2014:943162-943162. doi:10.1155/2014/943162
197. Punthakee Z, Goldenberg R, Katz P. Definition, Classification and Diagnosis of Diabetes, Prediabetes and Metabolic Syndrome. *Canadian Journal of Diabetes*. 2018;42:S10-S15. doi:10.1016/j.jcjd.2017.10.003
198. Neergaard-Petersen S, Hvas A-M, Grove EL, Larsen SB, Gregersen S, Kristensen SD. The Influence of Haemoglobin A1c Levels on Platelet Aggregation and Platelet Turnover in Patients with Coronary Artery Disease Treated with Aspirin. *PLOS ONE*. 2015;10(7):e0132629. doi:10.1371/journal.pone.0132629
199. Serruys PW, Silber S, Garg S, et al. Comparison of zotarolimus-eluting and everolimus-eluting coronary stents. *N Engl J Med*. Jul 8 2010;363(2):136-46. doi:10.1056/NEJMoa1004130
200. Mauri L, Hsieh W-h, Massaro JM, Ho KKL, D'Agostino R, Cutlip DE. Stent Thrombosis in Randomized Clinical Trials of Drug-Eluting Stents. *New England Journal of Medicine*. 2007/03/08 2007;356(10):1020-1029. doi:10.1056/NEJMoa067731
201. Pencina MJ, D'Agostino RB, Sr., D'Agostino RB, Jr., Vasan RS. Evaluating the added predictive ability of a new marker: from area under the ROC curve to reclassification and beyond. *Stat Med*. Jan 30 2008;27(2):157-72; discussion 207-12. doi:10.1002/sim.2929
202. Tofler GH, Massaro J, O'Donnell CJ, et al. Plasminogen activator inhibitor and the risk of cardiovascular disease: The Framingham Heart Study. *Thromb Res*. Apr 2016;140:30-35. doi:10.1016/j.thromres.2016.02.002
203. Jung RG, Simard T, Labinaz A, et al. Role of plasminogen activator inhibitor-1 in coronary pathophysiology. *Thromb Res*. Apr 2018;164:54-62. doi:10.1016/j.thromres.2018.02.135
204. Santos-Gallego CG, Bayón J, Badimón JJ. Thrombi of Different Pathologies: Implications for Diagnosis and Treatment. 2010;12(3):274-291. doi:10.1007/s11936-010-0075-8
205. Jung RG, Motazedian P, Ramirez FD, et al. Association between plasminogen activator inhibitor-1 and cardiovascular events: a systematic review and meta-analysis. *Thrombosis Journal*. 2018/06/05 2018;16(1):12. doi:10.1186/s12959-018-0166-4
206. Jung RG, Simard T, Di Santo P, et al. Performance of plasminogen activator inhibitor-1 as a biomarker in patients undergoing coronary angiography: Analytical and biological considerations. *Diab Vasc Dis Res*. Sep 2019;16(5):478-482. doi:10.1177/1479164119845123
207. Jung RG, Simard T, Di Santo P, et al. Performance of plasminogen activator inhibitor-1 as a biomarker in patients undergoing coronary angiography: Analytical and biological considerations. *Diabetes and Vascular Disease Research*. 2019:1479164119845123. doi:10.1177/1479164119845123
208. Lin DY, Wei LJ, Ying Z. Checking the Cox Model with Cumulative Sums of Martingale-Based Residuals. *Biometrika*. 1993;80(3):557-572. doi:10.2307/2337177
209. Stone GW, Kappetein AP, Sabik JF, et al. Five-Year Outcomes after PCI or CABG for Left Main Coronary Disease. *N Engl J Med*. Nov 7 2019;381(19):1820-1830. doi:10.1056/NEJMoa1909406
210. Juhan-Vague I, Pyke SD, Alessi MC, Jespersen J, Haverkate F, Thompson SG. Fibrinolytic factors and the risk of myocardial infarction or sudden death in patients with angina pectoris. ECAT Study Group. European Concerted Action on Thrombosis and Disabilities. *Circulation*. Nov 1 1996;94(9):2057-63. doi:10.1161/01.cir.94.9.2057
211. Redondo M, Carroll VA, Mauron T, et al. Hemostatic and fibrinolytic parameters in survivors of myocardial infarction: a low plasma level of plasmin-alpha2-antiplasmin complex is an independent predictor of coronary re-events. *Blood Coagul Fibrinolysis*. Jan 2001;12(1):17-24. doi:10.1097/00001721-200101000-00003

212. Ganti AK, Potti A, Yegnanarayan R. Plasma tissue plasminogen activator and plasminogen activator inhibitor-1 levels in acute myocardial infarction. *Pathophysiol Haemost Thromb*. Mar-Apr 2002;32(2):80-4. doi:10.1159/000065080
213. Kosaki R, Minoura Y, Ogura K, et al. Thrombomodulin can predict the incidence of second events in patients with acute coronary syndrome: Single-center, retrospective cohort study. *Journal of Cardiology*. 2018;72(6):494-500. doi:10.1016/j.jjcc.2018.05.006
214. Robinson SD, Ludlam CA, Boon NA, Newby DE. Endothelial fibrinolytic capacity predicts future adverse cardiovascular events in patients with coronary heart disease. *Arterioscler Thromb Vasc Biol*. Jul 2007;27(7):1651-6. doi:10.1161/ATVBAHA.107.143248
215. Pineda J, Marin F, Marco P, et al. The prognostic value of biomarkers after a premature myocardial infarction. *Int J Cardiol*. Sep 3 2010;143(3):249-54. doi:10.1016/j.ijcard.2009.02.019
216. Yano Y, Hoshide S, Shimada K, Kario K. The impact of cigarette smoking on 24-hour blood pressure, inflammatory and hemostatic activity, and cardiovascular risk in Japanese hypertensive patients. *J Clin Hypertens (Greenwich)*. Apr 2013;15(4):234-40. doi:10.1111/jch.12047
217. Yano Y, Nakazato M, Toshinai K, et al. Circulating Des-acyl Ghrelin Improves Cardiovascular Risk Prediction in Older Hypertensive Patients. 2014;27(5):727-733. doi:10.1093/ajh/hpt232
218. Vilahur G, Ben-Aicha S, Badimon L. New insights into the role of adipose tissue in thrombosis. *Cardiovascular Research*. 2017;113(9):1046-1054. doi:10.1093/cvr/cvx086
219. Shaw GJ, Dhamija A, Bavani N, Wagner KR, Holland CK. Arrhenius temperature dependence of in vitro tissue plasminogen activator thrombolysis. 2007;52(11):2953-2967. doi:10.1088/0031-9155/52/11/002
220. Alaigh P, Hoffman CJ, Korlipara G, et al. Lipoprotein(a) level does not predict restenosis after percutaneous transluminal coronary angioplasty. *Arterioscler Thromb Vasc Biol*. Aug 1998;18(8):1281-6. doi:10.1161/01.atv.18.8.1281
221. Inoue T, Yaguchi I, Mizoguchi K, et al. Accelerated plasminogen activator inhibitor may prevent late restenosis after coronary stenting in acute myocardial infarction. *Clin Cardiol*. Mar 2003;26(3):153-7. doi:10.1002/clc.4960260311
222. Nordt TK, Moser M, Kohler B, et al. Augmented platelet aggregation as predictor of reocclusion after thrombolysis in acute myocardial infarction. *Thromb Haemost*. Dec 1998;80(6):881-6.
223. Byrne RA, Joner M, Kastrati A. Stent thrombosis and restenosis: what have we learned and where are we going? The Andreas Grüntzig Lecture ESC 2014. *European Heart Journal*. 2015;36(47):3320-3331. doi:10.1093/eurheartj/ehv511
224. Christ G, Nikfardjam M, Huber-Beckmann R, et al. Predictive value of plasma plasminogen activator inhibitor-1 for coronary restenosis: dependence on stent implantation and antithrombotic medication. *Journal of Thrombosis and Haemostasis*. 2005/02/01 2005;3(2):233-239. doi:10.1111/j.1538-7836.2004.01062.x
225. Luo M, Ji Y, Luo Y, Li R, Fay WP, Wu J. Plasminogen activator inhibitor-1 regulates the vascular expression of vitronectin. *J Thromb Haemost*. Dec 2017;15(12):2451-2460. doi:10.1111/jth.13869
226. Muldowney James AS, Stringham John R, Levy Shawn E, et al. Antiproliferative Agents Alter Vascular Plasminogen Activator Inhibitor-1 Expression. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2007/02/01 2007;27(2):400-406. doi:10.1161/01.ATV.0000254677.12861.b8
227. Sambola A, Garcia Del Blanco B, Ruiz-Meana M, et al. Increased von Willebrand factor, P-selectin and fibrin content in occlusive thrombus resistant to lytic therapy. *Thromb Haemost*. Jun 2 2016;115(6):1129-37. doi:10.1160/TH15-12-0985
228. Chiva-Blanch G, Laake K, Myhre P, et al. Platelet-, monocyte-derived and tissue factor-carrying circulating microparticles are related to acute myocardial infarction severity. *PLOS ONE*. 2017;12(2):e0172558. doi:10.1371/journal.pone.0172558

229. Palmerini T, Benedetto U, Biondi-Zoccai G, et al. Long-Term Safety of Drug-Eluting and Bare-Metal Stents: Evidence From a Comprehensive Network Meta-Analysis. *Journal of the American College of Cardiology*. 2015/06/16/ 2015;65(23):2496-2507. doi:https://doi.org/10.1016/j.jacc.2015.04.017
230. Jung RG, Simard T, Di Santo P, et al. Evaluation of plasminogen activator inhibitor-1 as a biomarker of unplanned revascularization and major adverse cardiac events in coronary angiography and percutaneous coronary intervention. *Thrombosis Research*. 2020/04/24/ 2020;doi:https://doi.org/10.1016/j.thromres.2020.04.025
231. Luo M, Ji Y, Luo Y, Li R, Fay WP, Wu J. Plasminogen activator inhibitor-1 regulates the vascular expression of vitronectin. *Journal of Thrombosis and Haemostasis*. 2017;15(12):2451-2460. doi:10.1111/jth.13869
232. Suades R, Padró T, Crespo J, et al. Circulating microparticle signature in coronary and peripheral blood of ST elevation myocardial infarction patients in relation to pain-to-PCI elapsed time. *International Journal of Cardiology*. 2016/01/01/ 2016;202:378-387. doi:https://doi.org/10.1016/j.ijcard.2015.09.011
233. Horn P, Baars T, Kahlert P, et al. Release of Intracoronary Microparticles during Stent Implantation into Stable Atherosclerotic Lesions under Protection with an Aspiration Device. *PLOS ONE*. 2015;10(4):e0124904. doi:10.1371/journal.pone.0124904
234. De Paoli SH, Tegegn TZ, Elhelu OK, et al. Dissecting the biochemical architecture and morphological release pathways of the human platelet extracellular vesiculome. *Cellular and Molecular Life Sciences*. 2018;doi:10.1007/s00018-018-2771-6
235. Laffont B, Corduan A, Plé H, et al. Activated platelets can deliver mRNA regulatory Ago2•microRNA complexes to endothelial cells via microparticles. *Blood*. 2013;122(2):253-261. doi:10.1182/blood-2013-03-492801
236. Laffont B, Corduan A, Rousseau M, et al. Platelet microparticles reprogram macrophage gene expression and function. *Thromb Haemost*. Jan 2016;115(2):311-23. doi:10.1160/TH15-05-0389
237. Vajen T, Benedikter BJ, Heinzmann ACA, et al. Platelet extracellular vesicles induce a pro-inflammatory smooth muscle cell phenotype. *Journal of Extracellular Vesicles*. 2017/12/01 2017;6(1):1322454. doi:10.1080/20013078.2017.1322454
238. Duchez A-C, Boudreau LH, Naika GS, et al. Platelet microparticles are internalized in neutrophils via the concerted activity of 12-lipoxygenase and secreted phospholipase A2-IIA. *Proceedings of the National Academy of Sciences*. 2015;112(27):E3564-E3573. doi:10.1073/pnas.1507905112
239. Michael JV, Wurtzel JGT, Mao GF, et al. Platelet microparticles infiltrating solid tumors transfer miRNAs that suppress tumor growth. *Blood*. 2017;130(5):567-580. doi:10.1182/blood-2016-11-751099
240. Al Ghaithi R, Mori J, Nagy Z, et al. Evaluation of the Total Thrombus-Formation System (T-TAS): application to human and mouse blood analysis. *Platelets*. 2019;30(7):893-900. doi:10.1080/09537104.2018.1535704
241. Ito M, Kaikita K, Sueta D, et al. Total Thrombus-Formation Analysis System (T-TAS) Can Predict Periprocedural Bleeding Events in Patients Undergoing Catheter Ablation for Atrial Fibrillation. *Journal of the American Heart Association*. 5(1):e002744. doi:10.1161/JAHA.115.002744
242. Sueta D, Kaikita K, Okamoto N, et al. A novel quantitative assessment of whole blood thrombogenicity in patients treated with a non-vitamin K oral anticoagulant. *Int J Cardiol*. Oct 15 2015;197:98-100. doi:10.1016/j.ijcard.2015.06.041
243. Hosokawa K, Ohnishi T, Kondo T, et al. A novel automated microchip flow-chamber system to quantitatively evaluate thrombus formation and antithrombotic agents under blood flow conditions. *J Thromb Haemost*. Oct 2011;9(10):2029-37. doi:10.1111/j.1538-7836.2011.04464.x
244. Jung R, Radko S, Pelka P. The Dual Nature of Nek9 in Adenovirus Replication. *J Virol*. Feb 15 2016;90(4):1931-43. doi:10.1128/JVI.02392-15
245. Zeng Z, Xia L, Fan X, et al. Platelet-derived miR-223 promotes a phenotypic switch in arterial injury repair. *The Journal of Clinical Investigation*. 03/01/ 2019;129(3):1372-1386. doi:10.1172/JCI124508

246. Ruopp MD, Perkins NJ, Whitcomb BW, Schisterman EF. Youden Index and Optimal Cut-Point Estimated from Observations Affected by a Lower Limit of Detection. *Biometrical Journal*. 2008;50(3):419-430. doi:10.1002/bimj.200710415
247. Simone TM, Higgins SP, Archambeault J, et al. A small molecule PAI-1 functional inhibitor attenuates neointimal hyperplasia and vascular smooth muscle cell survival by promoting PAI-1 cleavage. *Cell Signal*. 2015;27(5):923-933. doi:10.1016/j.cellsig.2015.01.009
248. Kubala MH, Punj V, Placencio-Hickok VR, et al. Plasminogen Activator Inhibitor-1 Promotes the Recruitment and Polarization of Macrophages in Cancer. *Cell Rep*. 2018;25(8):2177-2191.e7. doi:10.1016/j.celrep.2018.10.082
249. Placencio VR, DeClerck YA. Plasminogen Activator Inhibitor-1 in Cancer: Rationale and Insight for Future Therapeutic Testing. *Cancer Res*. Aug 1 2015;75(15):2969-74. doi:10.1158/0008-5472.CAN-15-0876
250. Mohri H, Ohkubo T. How Vitronectin Binds to Activated Glycoprotein Ilb-IIIa Complex and Its Function in Platelet Aggregation. *American Journal of Clinical Pathology*. 1991;96(5):605-609. doi:10.1093/ajcp/96.5.605
251. Boulanger CM, Loyer X, Rautou PE, Amabile N. Extracellular vesicles in coronary artery disease. *Nat Rev Cardiol*. May 2017;14(5):259-272. doi:10.1038/nrcardio.2017.7
252. Merten M, Pakala R, Thiagarajan P, Benedict CR. Platelet Microparticles Promote Platelet Interaction With Subendothelial Matrix in a Glycoprotein IIb/IIIa Dependent Mechanism. 1999;99(19):2577-2582. doi:10.1161/01.cir.99.19.2577
253. Burger D, Schock S, Charlie, Augusto, Antoine, Rhian. Microparticles: biomarkers and beyond. *Clinical Science*. 2013;124(7):423-441. doi:10.1042/cs20120309
254. Shah R, Patel T, Freedman JE. Circulating Extracellular Vesicles in Human Disease. *New England Journal of Medicine*. 2018;379(10):958-966. doi:10.1056/NEJMra1704286
255. Suades R, Padro T, Vilahur G, Badimon L. Circulating and platelet-derived microparticles in human blood enhance thrombosis on atherosclerotic plaques. *Thromb Haemost*. Dec 2012;108(6):1208-19. doi:10.1160/TH12-07-0486
256. Nozaki T, Sugiyama S, Koga H, et al. Significance of a multiple biomarkers strategy including endothelial dysfunction to improve risk stratification for cardiovascular events in patients at high risk for coronary heart disease. *J Am Coll Cardiol*. Aug 11 2009;54(7):601-8. doi:10.1016/j.jacc.2009.05.022
257. Sandoval Y, Bielinski SJ, Daniels LB, et al. Atherosclerotic Cardiovascular Disease Risk Stratification Based on Measurements of Troponin and Coronary Artery Calcium. *Journal of the American College of Cardiology*. 2020;76(4):357-370. doi:10.1016/j.jacc.2020.05.057
258. Sinning JM, Losch J, Walenta K, Bohm M, Nickenig G, Werner N. Circulating CD31+/Annexin V+ microparticles correlate with cardiovascular outcomes. *Eur Heart J*. Aug 2011;32(16):2034-41. doi:10.1093/eurheartj/ehq478
259. Amabile N, Guérin AP, Leroyer A, et al. Circulating Endothelial Microparticles Are Associated with Vascular Dysfunction in Patients with End-Stage Renal Failure. *Journal of the American Society of Nephrology*. 2005;16(11):3381-3388. doi:10.1681/asn.2005050535
260. Boulanger CM, Amabile N, Tedgui A. Circulating microparticles: a potential prognostic marker for atherosclerotic vascular disease. *Hypertension*. Aug 2006;48(2):180-6. doi:10.1161/01.HYP.0000231507.00962.b5
261. Ji Y, Weng Z, Fish P, et al. Pharmacological Targeting of Plasminogen Activator Inhibitor-1 Decreases Vascular Smooth Muscle Cell Migration and Neointima Formation. *Arteriosclerosis, thrombosis, and vascular biology*. 2016;36(11):2167-2175. doi:10.1161/ATVBAHA.116.308344
262. Khoukaz HB, Ji Y, Braet DJ, et al. Drug Targeting of Plasminogen Activator Inhibitor-1 Inhibits Metabolic Dysfunction and Atherosclerosis in a Murine Model of Metabolic Syndrome. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2020;40(6):1479-1490. doi:doi:10.1161/ATVBAHA.119.313775

Appendix A

Methodological quality of COVID-19 clinical research

Citation information:

Jung RG, Di Santo P, Clifford C, Prosperi-Porta G, Skanes S, Hung A, Visintini S, Parlow S, Ramirez FD, Simard T, Hibbert B. Methodological quality of COVID-19 clinical research: a systematic review and matched cohort analysis. (**2021**). *Nature Communications*

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Author: Richard G. Jung et al

Publication: Nature Communications

Publisher: Springer Nature

Date: Feb 11, 2021

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nature COMMUNICATIONS

ARTICLE

https://doi.org/10.1038/s41467-021-21220-5 OPEN

Methodological quality of COVID-19 clinical research

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The COVID-19 pandemic began in early 2020 with major health consequences. While a need to disseminate information to the medical community and general public was paramount, concerns have been raised regarding the scientific rigor in published reports. We performed a systematic review to evaluate the methodological quality of currently available COVID-19 studies compared to historical controls. A total of 9895 titles and abstracts were screened and 686 COVID-19 articles were included in the final analysis. Comparative analysis of COVID-19 to historical articles reveals a shorter time to acceptance (13.0[IQR, 5.0–25.0] days vs. 110.0[IQR, 71.0–156.0] days in COVID-19 and control articles, respectively; $p < 0.0001$). Furthermore, methodological quality scores are lower in COVID-19 articles across all study designs. COVID-19 clinical studies have a shorter time to publication and have lower methodological quality scores than control studies in the same journal. These studies should be revisited with the emergence of stronger evidence.

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The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic spread globally in early 2020 with substantial health and economic consequences. This was associated with an exponential increase in scientific publications related to the coronavirus disease 2019 (COVID-19) in order to rapidly elucidate the natural history and identify diagnostic and therapeutic tools[1].

While a need to rapidly disseminate information to the medical community, governmental agencies, and general public was paramount—major concerns have been raised regarding the scientific rigor in the literature[2]. Poorly conducted studies may originate from failure at any of the four consecutive research stages: (1) choice of research question relevant to patient care, (2) quality of research design[3], (3) adequacy of publication, and (4) quality of research reports. Furthermore, evidence-based medicine relies on a hierarchy of evidence, ranging from the highest level of randomized controlled trials (RCT) to the lowest level of case series and case reports[4].

Given the implications for clinical care, policy decision making, and concerns regarding methodological and peer-review standards for COVID-19 research[5], we performed a formal evaluation of the methodological quality of published COVID-19 literature. Specifically, we undertook a systematic review to identify COVID-19 clinical literature and matched them to historical controls to formally evaluate the following: (1) the methodological quality of COVID-19 studies using established quality tools and checklists, (2) the methodological quality of COVID-19 studies, stratified by median time to acceptance, geographical regions, and journal impact factor and (3) a comparison of COVID-19 methodological quality to matched controls.

Herein, we show that COVID-19 articles are associated with lower methodological quality scores. Moreover, in a matched cohort analysis with control articles from the same journal, we reveal that COVID-19 articles are associated with lower quality scores and shorter time from submission to acceptance. Ultimately, COVID-19 clinical studies should be revisited with the emergence of stronger evidence.

Results

Article selection. A total of 14787 COVID-19 papers were identified as of May 14, 2020 and 4892 duplicate articles were removed. In total, 9895 titles and abstracts were screened, and 9101 articles were excluded due to the study being pre-clinical in nature, case report, case series <5 patients, in a language other than English, reviews (including systematic reviews), study protocols or methods, and other coronavirus variants with an overall inter-rater study inclusion agreement of 96.7% ($\kappa = 0.81$; 95% CI, 0.79–0.83). A total number of 794 full texts were reviewed for eligibility. Over 108 articles were excluded for ineligible study design or publication type (such as letter to the editors, editorials, case reports or case series <5 patients), wrong patient population, non-English language, duplicate articles, wrong outcomes and publication in a non-peer-reviewed journal. Ultimately, 686

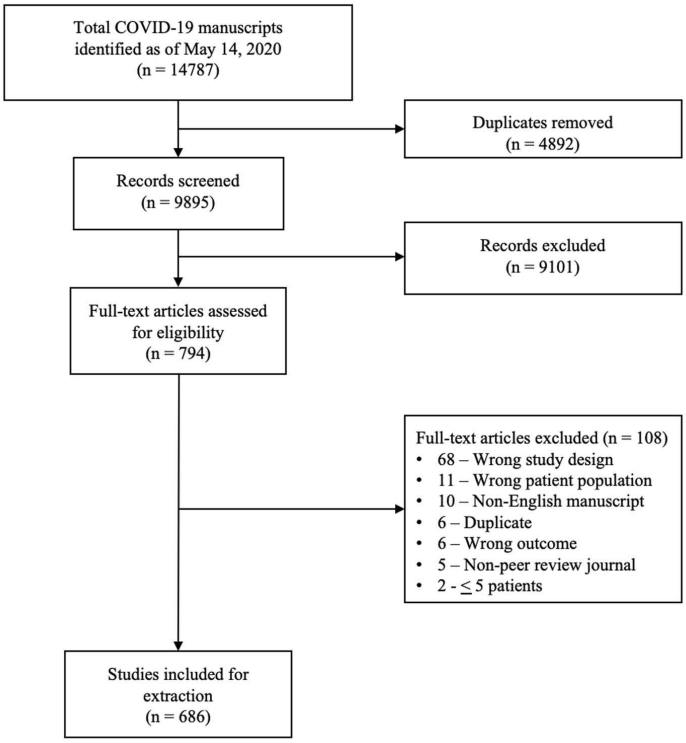


Fig. 1 Literature search and selection of COVID-19 articles. A total of 14787 articles were identified and 4892 duplicate articles were removed. Overall, 9895 articles were screened by title and abstract leaving 794 articles for full-text screening. Over 108 articles were excluded, leaving a total of 686 articles that underwent methodological quality assessment.

Table 1 Characteristics of COVID-19 clinical literature until May 14, 2020.

	COVID-19 articles ($n = 686$)	Matched articles ($n = 1078$)		*P*-value*
		COVID-19 ($n = 539$)	Control ($n = 539$)	
	N (%)	*N* (%)	*N* (%)	
Study design				
Case series	380 (55.4)	277 (51.4)	277 (51.4)	
Cohort	199 (29.0)	174 (32.3)	174 (32.3)	
Case control	38 (5.5)	32 (5.9)	32 (5.9)	
Diagnostic	63 (9.2)	53 (9.8)	53 (9.8)	
Randomized controlled trial	6 (0.9)	3 (0.6)	3 (0.6)	
Geographic region				<0.0001
Asia/Oceania	469 (68.4)	377 (69.9)	167 (31.0)	
Europe	139 (20.3)	99 (18.4)	176 (32.7)	
Americas	78 (11.4)	63 (11.7)	196 (36.4)	
Type of article				0.91
Original research	614 (89.5)	486 (90.2)	484 (89.8)	
Letter	35 (5.1)	26 (4.8)	29 (5.4)	
Communication	37 (5.4)	27 (5.0)	26 (4.8)	
Retrospective study	590 (86.0)	459 (85.2)	386 (71.6)	<0.0001
Sex reported	620 (90.4)	484 (89.8)	483 (89.6)	0.92
Sample size calculated without case series	($n = 306$)	($n = 262$)	($n = 262$)	
	7 (2.3)	4 (1.5)	30 (11.5)	<0.0001
Method of SARS-CoV-2 diagnosis				
PCR	548 (79.9)	437 (81.1)	—	—
ELISA	2 (0.3)	1 (0.2)	—	—
Physical exam only	5 (0.7)	4 (0.7)	—	—
Multiple	3 (0.4)	2 (0.4)	—	—
Unknown	128 (18.7)	95 (17.6)	—	—
Ethics approval				0.48
Received	556 (81.0)	433 (80.3)	451 (83.7)	
Not required/received	91 (13.3)	73 (13.5)	60 (11.1)	
Not mentioned	39 (5.7)	33 (6.1)	28 (4.1)	
Impact factor—median (IQR) ($n = 652$)	4.7 (2.9–7.6)	—	—	—
Time to acceptance—median (IQR) ($n = 450$)	13.0 (5.0–25.0)	13.0 (5.0–25.0)	110.0 (71.0–156.0)	<0.0001

COVID-19 articles were 1:1 matched with control articles from the same journal (identical impact factor) and study design. *PCR*: polymerase chain reaction; *ELISA*: enzyme-linked immunosorbent assay.
*Statistical analysis was conducted to compare COVID-19 to historical control articles with their corresponding *P*-values.

articles were identified with an inter-rater agreement of 86.5% ($\kappa = 0.68$; 95% CI, 0.67–0.70) (Fig. 1).

COVID-19 literature methodological quality. Most studies originated from Asia/Oceania with 469 (68.4%) studies followed by Europe with 139 (20.3%) studies, and the Americas with 78 (11.4%) studies. Of included studies, 380 (55.4%) were case series, 199 (29.0%) were cohort, 63 (9.2%) were diagnostic, 38 (5.5%) were case–control, and 6 (0.9%) were RCTs. Most studies (590, 86.0%) were retrospective in nature, 620 (90.4%) reported the sex of patients, and 7 (2.3%) studies excluding case series calculated their sample size a priori. The method of SARS-CoV-2 diagnosis was reported in 558 studies (81.3%) and ethics approval was obtained in 556 studies (81.0%). Finally, journal impact factor of COVID-19 manuscripts was 4.7 (IQR, 2.9–7.6) with a time to acceptance of 13.0 (IQR, 5.0–25.0) days (Table 1).

Overall, when COVID-19 articles were stratified by study design, a mean case series score (out of 5) (SD) of 3.3 (1.1), mean NOS cohort study score (out of 8) of 5.8 (1.5), mean NOS case–control study score (out of 8) of 5.5 (1.9), and low bias present in 4 (6.4%) diagnostic studies was observed (Table 2 and Fig. 2). Furthermore, in the 6 RCTs in the COVID-19 literature, there was a high risk of bias with little consideration for sequence generation, allocation concealment, blinding, incomplete outcome data, and selective outcome reporting (Table 2).

For secondary outcomes, rapid time from submission to acceptance (stratified by median time of acceptance of <13.0 days) was associated with lower methodological quality scores for case series and cohort study designs but not for case–control nor diagnostic studies (Fig. 3A–D). Low journal impact factor (<10) was associated with lower methodological quality scores for case series, cohort, and case–control designs (Fig. 3E–H). Finally, studies originating from different geographical regions had no differences in methodological quality scores with the exception of cohort studies (Fig. 3I–L). When dichotomized by high vs. low methodological quality scores, a similar trend was observed with rapid time from submission to acceptance (34.4% vs. 46.3%, $p = 0.01$, Supplementary Fig. 1B), low impact factor journals (<10) was associated with lower methodological quality score (38.8% vs. 68.0%, $p < 0.0001$, Supplementary Fig. 1C). Finally, studies originating in either Americas or Asia/Oceania was associated with higher methodological quality scores than Europe (Supplementary Fig. 1D).

Methodological quality score differences in COVID-19 versus historical control. We matched 539 historical control articles to COVID-19 articles from the same journal with identical study designs in the previous year for a final analysis of 1078 articles (Table 1). Overall, 554 (51.4%) case series, 348 (32.3%) cohort, 64 (5.9%) case–control, 106 (9.8%) diagnostic and 6 (0.6%) RCTs were identified from the 1078 total articles. Differences exist between COVID-19 and historical control articles in geographical region of publication, retrospective study design, and sample size calculation (Table 1). Time of acceptance was 13.0

Table 2 Methodological quality scores of different study types stratified by COVID-19 and historical control articles.

	All COVID-19 ($n = 686$)	COVID-19 ($n = 539$)	Control ($n = 539$)	*P*-value*
Case series	($n = 380$)	($n = 277$)	($n = 277$)	
Overall score—mean (SD)	3.3 (1.1)	3.3 (1.1)	4.3 (0.8)	<0.0001
Selection	172 (45%)	115 (42%)	171 (62%)	
Ascertainment of exposure	284 (75%)	210 (76%)	258 (93%)	
Ascertainment of outcome	304 (80%)	224 (81%)	258 (93%)	
Outcome—was follow-up long enough	215 (57%)	159 (57%)	268 (97%)	
Reporting—sufficient detail provided	289 (76%)	210 (76%)	235 (85%)	
Case-control study	($n = 38$)	($n = 32$)	($n = 32$)	
Overall score—mean (SD)	5.5 (1.9)	5.4 (1.9)	6.6 (1.0)	0.0027
Selection—case definition	22 (58%)	16 (50%)	21 (66%)	
Selection—representativeness of cases	26 (68%)	22 (69%)	30 (94%)	
Selection—controls	20 (53%)	16 (50%)	19 (59%)	
Selection—definition of controls	25 (66%)	20 (63%)	29 (91%)	
Comparability	25 (66%)	22 (69%)	26 (81%)	
Exposure—ascertainment	31 (82%)	26 (81%)	29 (91%)	
Exposure—same method of ascertainment between cases and controls	33 (87%)	28 (88%)	31 (97%)	
Exposure—non-response rate	27 (71%)	22 (69%)	26 (81%)	
Cohort study	($n = 199$)	($n = 174$)	($n = 174$)	
Overall score—mean (SD)	5.8 (1.5)	5.8 (1.6)	7.1 (1.0)	<0.0001
Selection—representativeness of cases	145 (73%)	126 (72%)	152 (87%)	
Selection—non-exposed cohort	161 (81%)	140 (81%)	165 (95%)	
Selection—ascertainment of exposure	159 (80%)	138 (79%)	161 (93%)	
Selection—outcome not present at the beginning	106 (53%)	92 (53%)	131 (75%)	
Comparability	137 (69%)	120 (69%)	140 (81%)	
Outcome—ascertainment	177 (89%)	155 (89%)	162 (93%)	
Outcome—was follow-up long enough	113 (57%)	98 (56%)	161 (93%)	
Outcome—adequacy of follow-up of cohort	163 (82%)	142 (82%)	155 (89%)	
Diagnostic study	($n = 63$)	($n = 53$)	($n = 53$)	
Risk of bias—low bias				
Patient selection	13 (21%)	11 (21%)	23 (43%)	
Index test	13 (21%)	9 (17%)	31 (59%)	
Reference standard	46 (73%)	40 (76%)	39 (74%)	
Flow and timing	41 (65%)	36 (68%)	41 (77%)	
Overall (low bias)	4 (6%)	12 (23%)	24 (45%)	0.02
Applicability concerns—low bias				
Patient selection	56 (89%)	49 (93%)	51 (96%)	
Index test	60 (95%)	50 (94%)	51 (96%)	
Reference standard	57 (91%)	47 (89%)	53 (100%)	
Overall (low bias)	52 (83%)	45 (85%)	50 (94%)	0.11
Randomized controlled trial	($n = 6$)	($n = 3$)	($n = 3$)	
Sequence generation risk of bias	3 (50%)	2 (67%)	2 (67%)	
Allocation concealment	2 (33%)	1 (33%)	3 (100%)	
Blinding of participants and personnel to all outcomes	1 (17%)	1 (33%)	2 (67%)	
Blinding outcome assessors for all outcomes	1 (17%)	1 (33%)	3 (100%)	
Incomplete outcome data for all outcomes	3 (50%)	2 (67%)	2 (67%)	
Selective outcome reporting	5 (83%)	3 (100%)	3 (100%)	
Other sources of bias	2 (33%)	1 (33%)	2 (67%)	

*Statistical analysis was conducted to compare COVID-19 to historical control articles with their corresponding *P*-values.

(IQR, 5.0–25.0) days in COVID-19 articles vs. 110.0 (IQR, 71.0–156.0) days in control articles (Table 1 and Fig. 4A, $p < 0.0001$). Case-series methodological quality score was lower in COVID-19 articles compared to the historical control (3.3 (1.1) vs. 4.3 (0.8); $n = 554$; $p < 0.0001$; Table 2 and Fig. 4B). Furthermore, NOS score was lower in COVID-19 cohort studies (5.8 (1.6) vs. 7.1 (1.0); $n = 348$; $p < 0.0001$; Table 2 and Fig. 4C) and case–control studies (5.4 (1.9) vs. 6.6 (1.0); $n = 64$; $p = 0.003$; Table 2 and Fig. 4D). Finally, lower risk of bias in diagnostic studies was in 12 COVID-19 articles (23%; $n = 53$) compared to 24 control articles (45%; $n = 53$; $p = 0.02$; Table 2 and Fig. 4E). A similar trend was observed between COVID-19 and historical control articles when dichotomized by good vs. low methodological quality scores (Supplementary Fig. 2).

Discussion

In this systematic evaluation of methodological quality, COVID-19 clinical research was primarily observational in nature with modest methodological quality scores. Not only were the study designs low in the hierarchy of scientific evidence, we found that COVID-19 articles were associated with a lower methodological quality scores when published with a shorter time of publication and in lower impact factor journals. Furthermore, in a matched cohort analysis with historical control articles identified from the same journal of the same study design, we demonstrated that COVID-19 articles were associated with lower quality scores and shorter time from submission to acceptance.

The present study demonstrates comparative differences in methodological quality scores between COVID-19 literature and

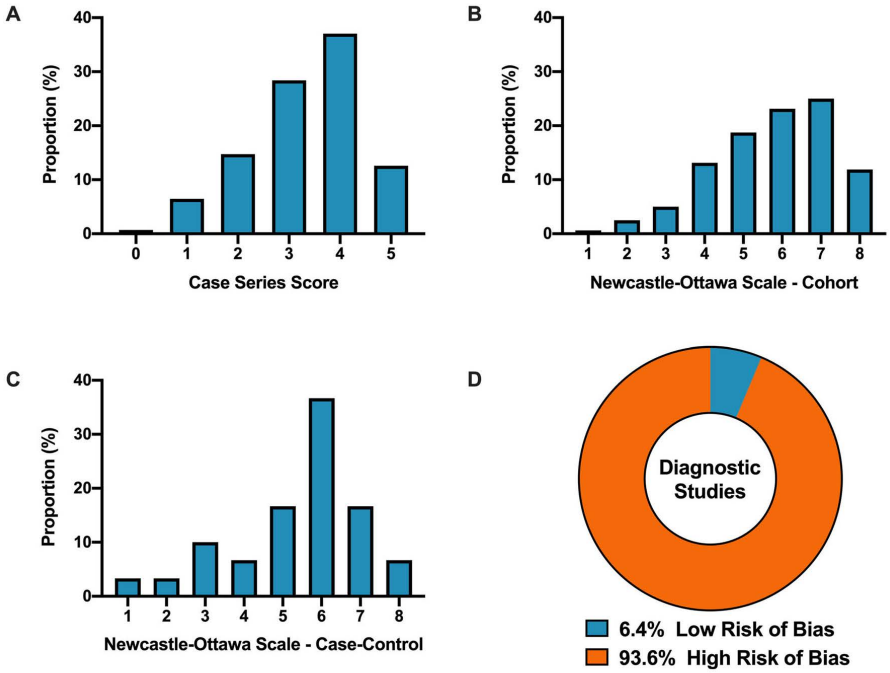


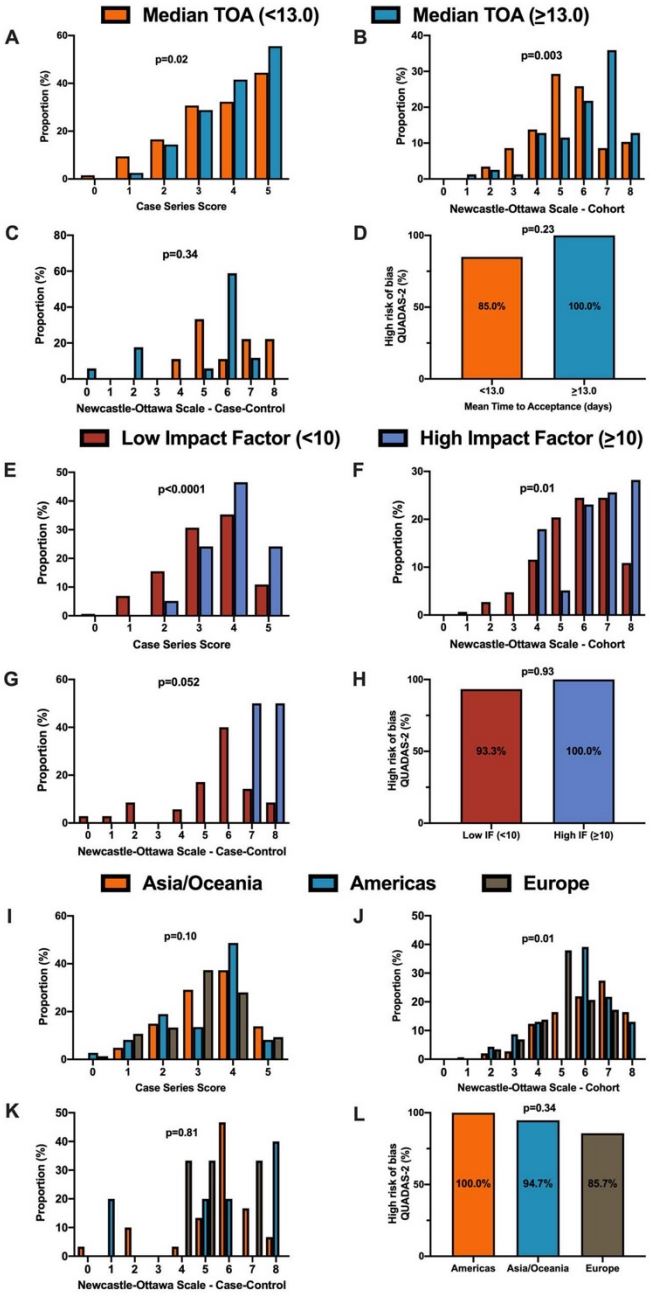
Fig. 2 COVID-19 clinical literature quality assessment. A Distribution of COVID-19 case series studies scored using the Murad tool ($n = 380$). **B** Distribution of COVID-19 cohort studies scored using the Newcastle–Ottawa Scale ($n = 199$). **C** Distribution of COVID-19 case-control studies scored using the Newcastle–Ottawa Scale ($n = 38$). **D** Distribution of COVID-19 diagnostic studies scored using the QUADAS-2 tool ($n = 63$). In panel **D**, blue represents low risk of bias and orange represents high risk of bias.

historical control articles. Overall, the accelerated publication of COVID-19 research was associated with lower study quality scores compared to previously published historical control studies. Our research highlights major differences in study quality between COVID-19 and control articles, possibly driven in part by a combination of more thorough editorial and/or peer-review process as suggested by the time to publication, and robust study design with questions which are pertinent for clinicians and patient management[3,6–11].

In the early stages of the COVID-19 pandemic, we speculate that an urgent need for scientific data to inform clinical, social and economic decisions led to shorter time to publication and explosion in publication of COVID-19 studies in both traditional peer-reviewed journals and preprint servers[1,12]. The accelerated scientific process in the COVID-19 pandemic allowed a rapid understanding of natural history of COVID-19 symptomology and prognosis, identification of tools including RT-PCR to diagnose SARS-CoV-2[13], and identification of potential therapeutic options such as tocilizumab and convalescent plasma which laid the foundation for future RCTs[14–16]. A delay in publication of COVID-19 articles due to a slower peer-review process may potentially delay dissemination of pertinent information against the pandemic. Despite concerns of slow peer review, major landmark trials (i.e. RECOVERY and ACTT-1 trial)[17,18] published their findings in preprint servers and media releases to allow for rapid dissemination. Importantly, the data obtained in these initial studies should be revisited as stronger data emerges as lower quality studies may fundamentally risk patient safety, resource allocation and future scientific research[19].

Unfortunately, poor evidence begets poor clinical decisions[20]. Furthermore, lower quality scientific evidence potentially undermines the public's trust in science during this time and has been evident through misleading information and high-profile retractions[12,21–23]. For example, the benefits of hydroxychloroquine, which were touted early in the pandemic based on limited data, have subsequently failed to be replicated in multiple observational studies and RCTs[5,24–30]. One poorly designed study combined with rapid publication led to considerable investment of both the scientific and medical community—akin to quinine being sold to the public as a miracle drug during the 1918 Spanish Influenza[31,32]. Moreover, as of June 30, 2020, ClinicalTrials.gov listed an astonishing 230 COVID-19 trials with hydroxychloroquine/plaquenil, and a recent living systematic review of observational studies and RCTs of hydroxychloroquine or chloroquine for COVID-19 demonstrated no evidence of benefit nor harm with concerns of severe methodological flaws in the included studies[33].

Our study has important limitations. We evaluated the methodological quality of existing studies using established checklists and tools. While it is tempting to associate methodological quality scores with reproducibility or causal inferences of the intervention, it is not possible to ascertain the impact on the study design and conduct of research nor results or conclusions in the identified reports[34]. Second, although the methodological quality scales and checklists used for the manuscript are commonly used for quality assessment in systematic reviews and meta-analyses[35–38], they can only assess the methodology without consideration for causal language and are prone to limitations[39,40]. Other tools such as the ROBINS-I and GRADE



exist to evaluate methodological quality of identified manuscripts, although no consensus currently exists for critical appraisal of non-randomized studies[41–43]. Furthermore, other considerations of quality such as sample size calculation, sex reporting or ethics approval are not considered in these quality scores. As such, the quality scores measured using these checklists only reflect the patient selection, comparability, diagnostic reference standard and methods to ascertain the outcome of the study. Third, the 1:1

Fig. 3 Differences in methodological quality scores in COVID-19 by secondary outcomes. A When stratified by time of acceptance (13.0 days), increased time of acceptance was associated with higher case series score ($n = 186$ for <13 days and $n = 193$ for >=13 days; $p = 0.02$). **B** Increased time of acceptance was associated with higher NOS cohort score ($n = 112$ for <13 days and $n = 144$ for >=13 days; $p = 0.003$). **C** No difference in time of acceptance and case–control score was observed ($n = 18$ for <13 days and $n = 27$ for >=13 days; $p = 0.34$). **D** No difference in time of acceptance and diagnostic risk of bias (QUADAS-2) was observed ($n = 43$ for <13 days and $n = 33$ for >=13 days; $p = 0.23$). **E** When stratified by impact factor (IF ≥10), high IF was associated with higher case series score ($n = 466$ for low IF and $n = 60$ for high IF; $p < 0.0001$). **F** High IF was associated with higher NOS cohort score ($n = 262$ for low IF and $n = 68$ for high IF; $p = 0.01$). **G** No difference in IF and case–control score was observed ($n = 62$ for low IF and $n = 2$ for high IF; $p = 0.052$). **H** No difference in IF and QUADAS-2 was observed ($n = 101$ for low IF and $n = 2$ for high IF; $p = 0.93$). **I** When stratified by geographical region, no difference in geographical region and case series score was observed ($n = 276$ Asia/Oceania, $n = 135$ Americas, and $n = 143$ Europe/Africa; $p = 0.10$). **J** Geographical region was associated with differences in cohort score ($n = 177$ Asia/Oceania, $n = 81$ Americas, and $n = 89$ Europe/Africa; $p = 0.01$). **K** No difference in geographical region and case–control score was observed ($n = 37$ Asia/Oceania, $n = 13$ Americas, and $n = 14$ Europe/Africa; $p = 0.81$). **L** No difference in geographical region and QUADAS-2 was observed ($n = 49$ Asia/Oceania, $n = 28$ Americas, and $n = 28$ Europe/Africa; $p = 0.34$). In panels **A**–**D**, orange represents lower median time of acceptance and blue represents high median time of acceptance. In panels **E**–**H**, red is low impact factor and blue is high impact factor. In panels **I**–**L**, orange represents Asia/Oceania, blue represents Americas, and brown represents Europe. Differences in distributions were analysed by two-sided Kruskal–Wallis test. Differences in diagnostic risk of bias were quantified by Chi-squares test. $p < 0.05$ was considered statistically significant.

ratio to identify our historical control articles may affect the precision estimates of our findings. Interestingly, a simulation of an increase from 1:1 to 1:4 control ratio tightened the precision estimates but did not significantly alter the point estimate[44]. Furthermore, the decision for 1:1 ratio in our study exists due to limitations of available historical control articles from the identical journal in the restricted time period combined with a large effect size and sample size in the analysis. Finally, our analysis includes early publications on COVID-19 and there is likely to be an improvement in quality of related studies and study design as the field matures and higher-quality studies. Accordingly, our findings are limited to the early body of research as it pertains to the pandemic and it is likely that over time research quality will improve over time.

In summary, the early body of peer-reviewed COVID-19 literature was composed primarily of observational studies that underwent shorter peer-review evaluation and were associated with lower methodological quality scores than comparable studies. COVID-19 clinical studies should be revisited with the emergence of stronger evidence.

Methods

A systematic literature search was conducted on May 14, 2020 (registered on June 3, 2020 at PROSPERO: CRD42020187318) and reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses. Furthermore, the cohort study was reported according to the Strengthening The Reporting of Observational Studies in Epidemiology checklist. The data supporting the findings of this study is available as Supplementary Data 1–2.

Data sources and searches. The search was created in MEDLINE by a medical librarian with expertise in systematic reviews (S.V.) using a combination of key terms and index headings related to COVID-19 and translated to the remaining bibliographic databases (Supplementary Tables 1–3). The searches were conducted in MEDLINE (Ovid MEDLINE(R) ALL 1946–), Embase (Ovid Embase Classic + Embase 1947–) and the Cochrane Central Register of Controlled Trials (from inception). Search results were limited to English-only publications, and a publication date limit of January 1, 2019 to present was applied. In addition, a Canadian Agency for Drugs and Technologies in Health search filter was applied in MEDLINE and Embase to remove animal studies, and commentary, newspaper article, editorial, letter and note publication types were also eliminated. Search results were exported to Covidence (Veritas Health Innovation, Melbourne, Australia) and duplicates were eliminated using the platform's duplicate identification feature.

Study selection, data extraction and methodological quality assessment. We included all types of COVID-19 clinical studies, including case series, observational studies, diagnostic studies and RCTs. For diagnostic studies, the reference standard for COVID-19 diagnosis was defined as a nasopharyngeal swab followed by reverse transcriptase-polymerase chain reaction in order to detect SARS-CoV-2. We excluded studies that were exploratory or pre-clinical in nature (i.e. in vitro or animal studies), case reports or case series of <5 patients, studies published in a language other than English, reviews, methods or protocols, and other coronavirus variants such as the Middle East respiratory syndrome.

The review team consisted of trained research staff with expertise in systematic reviews and one trainee. Title and abstracts were evaluated by two independent reviewers using Covidence and all discrepancies were resolved by consensus. Articles that were selected for full review were independently evaluated by two reviewers for quality assessment using a standardized case report form following the completion of a training period where all reviewers were trained with the original manuscripts which derived the tools or checklists along with examples for what were deemed high scores[35–38]. Following this, reviewers completed thirty full-text extractions and the two reviewers had to reach consensus and the process was repeated for the remaining manuscripts independently. When two independent reviewers were not able reach consensus, a third reviewer (principal investigator) provided oversight in the process to resolve the conflicted scores.

First and corresponding author names, date of publication, title of manuscript and journal of publication were collected for all included full-text articles. Journal impact factor was obtained from the 2018 InCites Journal Citation Reports from Clarivate Analytics. Submission and acceptance dates were collected in manuscripts when available. Other information such as study type, prospective or retrospective study, sex reporting, sample size calculation, method of SARS-CoV-2 diagnosis and ethics approval was collected by the authors. Methodological quality assessment was conducted using the Newcastle–Ottawa Scale (NOS) for case–control and cohort studies[37], QUADAS-2 tool for diagnostic studies[38], Cochrane risk of bias for RCTs[35] and a score derived by Murad et al. for case series studies[36].

Identification of historical control from identified COVID-19 articles. Following the completion of full-text extraction of COVID-19 articles, we obtained a historical control group by identifying reports matched in a 1:1 fashion. From the eligible COVID-19 article, historical controls were identified by searching the same journal in a systematic fashion by matching the same study design ("case series", "cohort", "case control" or "diagnostic") starting in the journal edition 12 months prior to the COVID-19 article publication on the publisher website (i.e. COVID-19 article published on April 2020, going backwards to April 2019) and proceeding forward (or backward if a specific article type was not identified) in a temporal fashion until the first matched study was identified following abstract screening by two independent reviewers. If no comparison article was found by either reviewers, the corresponding COVID-19 article was excluded from the comparison analysis. Following the identification of the historical control, data extraction and quality assessment was conducted on the identified articles using the standardized case report forms by two independent reviewers and conflicts resolved by consensus. The full dataset has been made available as Supplementary Data 1–2.

Data synthesis and statistical analysis. Continuous variables were reported as mean (SD) or median (IQR) as appropriate, and categorical variables were reported as proportions (%). Continuous variables were compared using Student t-test or Mann–Whitney U-test and categorical variables including quality scores were compared by χ^2, Fisher's exact test, or Kruskal–Wallis test.

The primary outcome of interest was to evaluate the methodological quality of COVID-19 clinical literature by study design using the Newcastle–Ottawa Scale (NOS) for case–control and cohort studies, QUADAS-2 tool for diagnostic studies[38], Cochrane risk of bias for RCTs[35], and a score derived by Murad et al. for case series studies[36]. Pre-specified secondary outcomes were comparison of methodological quality scores of COVID-19 articles by (i) median time to acceptance, (ii) impact factor, (iii) geographical region and (iv) historical comparator. Time of acceptance was defined as the time between submission to acceptance which captures peer review and editorial decisions. Geographical region

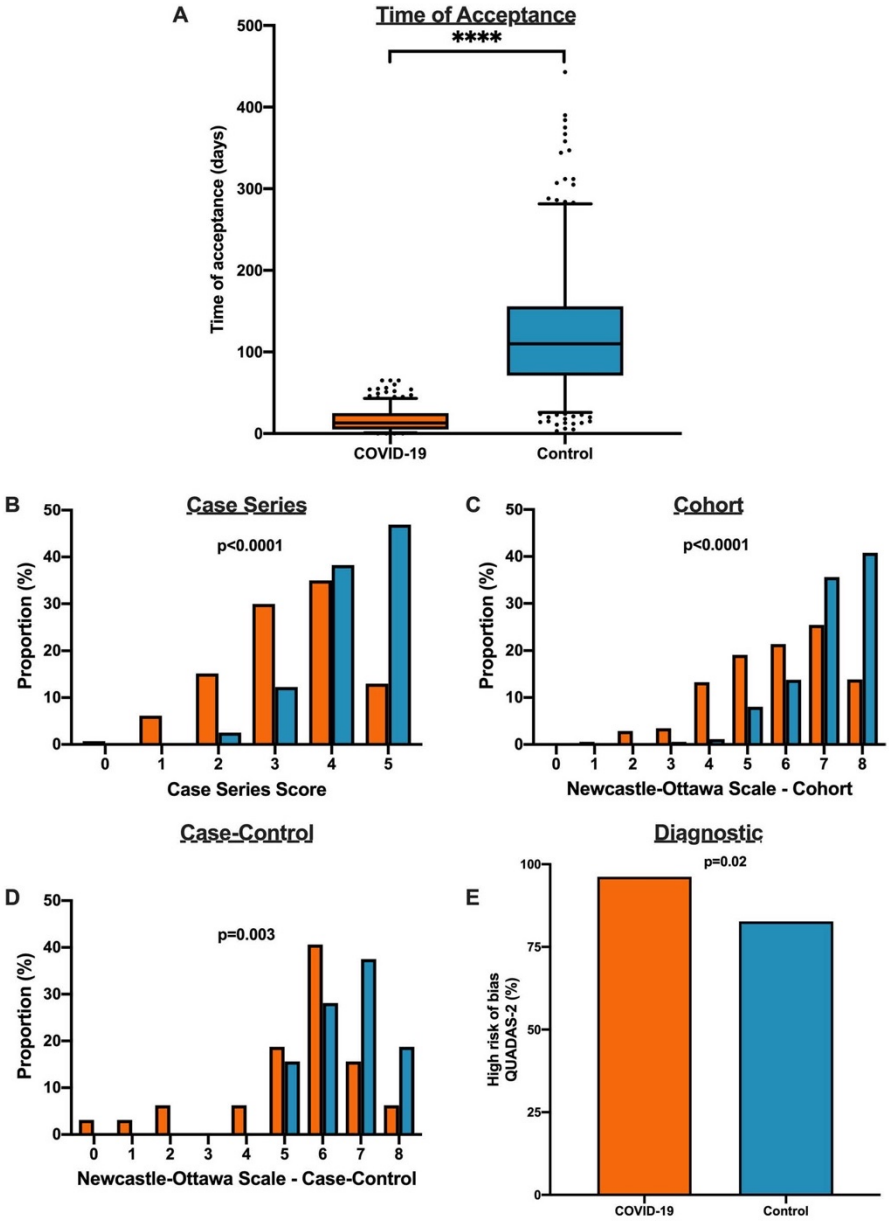


Fig. 4 Differences in methodological quality scores in COVID-19 compared to historical control articles. A Time to acceptance was reduced in COVID-19 articles compared to control articles (13.0 [IQR, 5.0–25.0] days vs. 110.0 [IQR, 71.0–156.0] days, $n = 347$ for COVID-19 and $n = 414$ for controls; $p < 0.0001$). **B** When compared to historical control articles, COVID-19 articles were associated with lower case series score ($n = 277$ for COVID-19 and $n = 277$ for controls; $p < 0.0001$). **C** COVID-19 articles were associated with lower NOS cohort score compared to historical control articles ($n = 174$ for COVID-19 and $n = 174$ for controls; $p < 0.0001$). **D** COVID-19 articles were associated with lower NOS case–control score compared to historical control articles ($n = 32$ for COVID-19 and $n = 32$ for controls; $p = 0.003$). **E** COVID-19 articles were associated with higher diagnostic risk of bias (QUADAS-2) compared to historical control articles ($n = 53$ for COVID-19 and $n = 53$ for controls; $p = 0.02$). For panel **A**, boxplot captures 5, 25, 50, 75 and 95% from the first to last whisker. Orange represents COVID-19 articles and blue represents control articles. Two-sided Mann–Whitney U-test was conducted to evaluate differences in time to acceptance between COVID-19 and control articles. Differences in study quality scores were evaluated by two-sided Kruskal–Wallis test. Differences in diagnostic risk of bias were quantified by Chi-squares test. $p < 0.05$ was considered statistically significant.

was stratified into continents including Asia/Oceania, Europe/Africa and Americas (North and South America). Post hoc comparison analysis between COVID-19 and historical control article quality scores were evaluated using Kruskal–Wallis test. Furthermore, good quality of NOS was defined as 3+ on selection and 1+ on comparability, and 2+ on outcome/exposure domains and high-quality case series scores was defined as a score ≥3.5. Due to a small sample size of identified RCTs, they were not included in the comparison analysis.

The finalized dataset was collected on Microsoft Excel v16.44. All statistical analyses were performed using SAS v9.4 (SAS Institute, Inc., Cary, NC, USA). Statistical significance was defined as $P < 0.05$. All figures were generated using GraphPad Prism v8 (GraphPad Software, La Jolla, CA, USA).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The authors can confirm that all relevant data are included in the paper and in Supplementary Data 1–2. The original search was conducted on MEDLINE, Embase and Cochrane Central Register of Controlled Trials.

Received: 16 July 2020; Accepted: 13 January 2021;
Published online: 11 February 2021

References

1. Chen, Q., Allot, A. & Lu, Z. Keep up with the latest coronavirus research. *Nature* **579**, 193 (2020).
2. Mahase, E. Covid-19: 146 researchers raise concerns over chloroquine study that halted WHO trial. *BMJ* https://doi.org/10.1136/bmj.m2197 (2020).
3. Chalmers, I. & Glasziou, P. Avoidable waste in the production and reporting of research evidence. *Lancet* **374**, 86–89 (2009).
4. Burns, P. B., Rohrich, R. J. & Chung, K. C. The levels of evidence and their role in evidence-based medicine. *Plast. Reconstr. Surg.* **128**, 305–310 (2011).
5. Alexander, P. E. et al. COVID-19 coronavirus research has overall low methodological quality thus far: case in point for chloroquine/hydroxychloroquine. *J. Clin. Epidemiol.* **123**, 120–126 (2020).
6. Barakat, A. P., Shokr, M., Ibrahim, J., Mandrola, J. & Elgendy, I. Y. Timeline from receipt to online publication of COVID-19 original research articles. Preprint at *medRxiv* https://doi.org/10.1101/2020.06.22.20137653 (2020).
7. Chan, A.-W. et al. Increasing value and reducing waste: addressing inaccessible research. *Lancet* **383**, 257–266 (2014).
8. Ioannidis, J. P. A. et al. Increasing value and reducing waste in research design, conduct, and analysis. *Lancet* **383**, 166–175 (2014).
9. Chalmers, I. et al. How to increase value and reduce waste when research priorities are set. *Lancet* **383**, 156–165 (2014).
10. Salman, R. A.-S. et al. Increasing value and reducing waste in biomedical research regulation and management. *Lancet* **383**, 176–185 (2014).
11. Glasziou, P. et al. Reducing waste from incomplete or unusable reports of biomedical research. *Lancet* **383**, 267–276 (2014).
12. Bauchner, H. The rush to publication: an editorial and scientific mistake. *JAMA* **318**, 1109–1110 (2017).
13. He, X. et al. Temporal dynamics in viral shedding and transmissibility of COVID-19. *Nat. Med.* **26**, 672–675 (2020).
14. Guaraldi, G. et al. Tocilizumab in patients with severe COVID-19: a retrospective cohort study. *Lancet Rheumatol.* **2**, e474–e484 (2020).
15. Duan, K. et al. Effectiveness of convalescent plasma therapy in severe COVID-19 patients. *Proc. Natl Acad. Sci. USA* **117**, 9490–9496 (2020).
16. Shen, C. et al. Treatment of 5 critically Ill patients with COVID-19 with convalescent plasma. *JAMA* **323**, 1582–1589 (2020).
17. Beigel, J. H. et al. Remdesivir for the treatment of covid-19—final report. *N. Engl. J. Med.* **383**, 1813–1826 (2020).
18. Group, R. C. et al. Dexamethasone in hospitalized patients with Covid-19—preliminary report. *N. Engl. J. Med.* https://doi.org/10.1056/NEJMoa2021436 (2020).
19. Ramirez, F. D. et al. Methodological rigor in preclinical cardiovascular studies: targets to enhance reproducibility and promote research translation. *Circ. Res* **120**, 1916–1926 (2017).
20. Heneghan, C. et al. Evidence based medicine manifesto for better healthcare. *BMJ* **357**, j2973 (2017).
21. Mehra, M. R., Desai, S. S., Ruschitzka, F. & Patel, A. N. RETRACTED: hydroxychloroquine or chloroquine with or without a macrolide for treatment of COVID-19: a multinational registry analysis. *Lancet* https://doi.org/10.1016/S0140-6736(20)31180-6 (2020).
22. Servick, K. & Enserink, M. The pandemic's first major research scandal erupts. *Science* **368**, 1041–1042 (2020).
23. Mehra, M. R., Desai, S. S., Kuy, S., Henry, T. D. & Patel, A. N. Retraction: Cardiovascular disease, drug therapy, and mortality in Covid-19. *N. Engl. J. Med.* **382**, 2582–2582, https://doi.org/10.1056/NEJMoa2007621. (2020).
24. Boulware, D. R. et al. A randomized trial of hydroxychloroquine as postexposure prophylaxis for Covid-19. *N. Engl. J. Med.* **383**, 517–525 (2020).
25. Gautret, P. et al. Clinical and microbiological effect of a combination of hydroxychloroquine and azithromycin in 80 COVID-19 patients with at least a six-day follow up: a pilot observational study. *Travel Med. Infect. Dis.* **34**, 101663–101663 (2020).
26. Geleris, J. et al. Observational study of hydroxychloroquine in hospitalized patients with Covid-19. *N. Engl. J. Med.* **382**, 2411–2418 (2020).
27. Borba, M. G. S. et al. Effect of high vs low doses of chloroquine diphosphate as adjunctive therapy for patients hospitalized with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection: a randomized clinical trial. *JAMA Netw. Open* **3**, e208857–e208857 (2020).
28. Mercuro, N. J. et al. Risk of QT interval prolongation associated with use of hydroxychloroquine with or without concomitant azithromycin among hospitalized patients testing positive for coronavirus disease 2019 (COVID-19). *JAMA Cardiol.* **5**, 1036–1041 (2020).
29. Molina, J. M. et al. No evidence of rapid antiviral clearance or clinical benefit with the combination of hydroxychloroquine and azithromycin in patients with severe COVID-19 infection. *Médecine et. Maladies Infectieuses* **50**, 384 (2020).
30. Group, R. C. et al. Effect of hydroxychloroquine in hospitalized patients with Covid-19. *N. Engl. J. Med.* 383, 2030–2040 (2020).
31. Shors, T. & McFadden, S. H. 1918 influenza: a Winnebago County, Wisconsin perspective. *Clin. Med. Res.* **7**, 147–156 (2009).
32. Stolberg, S. *A Mad Scramble to Stock Millions of Malaria Pills, Likely for Nothing* (The New York Times, 2020).
33. Hernandez, A. V., Roman, Y. M., Pasupuleti, V., Barboza, J. J. & White, C. M. Hydroxychloroquine or chloroquine for treatment or prophylaxis of COVID-19: a living systematic review. *Ann. Int. Med.* **173**, 287–296 (2020).
34. Glasziou, P. & Chalmers, I. Research waste is still a scandal—an essay by Paul Glasziou and Iain Chalmers. *BMJ* **363**, k4645 (2018).
35. Higgins, J. P. T. et al. The Cochrane Collaboration's tool for assessing risk of bias in randomised trials. *BMJ* **343**, d5928 (2011).
36. Murad, M. H., Sultan, S., Haffar, S. & Bazerbachi, F. Methodological quality and synthesis of case series and case reports. *BMJ Evid. Based Med.* **23**, 60–63 (2018).
37. Wells, G. S. B. et al. The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomised studies in meta-analysis. http://wwwohrica/programs/clinical_epidemiology/oxfordasp (2004).
38. Whiting, P. F. et al. QUADAS-2: a revised tool for the quality assessment of diagnostic accuracy studies. *Ann. Intern. Med.* **155**, 529–536 (2011).
39. Sanderson, S., Tatt, I. D. & Higgins, J. P. Tools for assessing quality and susceptibility to bias in observational studies in epidemiology: a systematic review and annotated bibliography. *Int. J. Epidemiol.* **36**, 666–676 (2007).
40. Stang, A. Critical evaluation of the Newcastle-Ottawa scale for the assessment of the quality of nonrandomized studies in meta-analyses. *Eur. J. Epidemiol.* **25**, 603–605 (2010).
41. Guyatt, G. et al. GRADE guidelines: 1. Introduction-GRADE evidence profiles and summary of findings tables. *J. Clin. Epidemiol.* **64**, 383–394 (2011).
42. Quigley, J. M., Thompson, J. C., Halfpenny, N. J. & Scott, D. A. Critical appraisal of nonrandomized studies-A review of recommended and commonly used tools. *J. Evaluation Clin. Pract.* **25**, 44–52 (2019).
43. Sterne, J. A. et al. ROBINS-I: a tool for assessing risk of bias in non-randomised studies of interventions. *BMJ* **355**, i4919 (2016).
44. Hamajima, N. et al. Case-control studies: matched controls or all available controls? *J. Clin. Epidemiol.* **47**, 971–975 (1994).

Acknowledgements

This study received no specific funding or grant from any agency in the public, commercial, or not-for-profit sectors. R.G.J. was supported by the Vanier CIHR Canada Graduate Scholarship. F.D.R. was supported by a CIHR Banting Postdoctoral Fellowship and a Royal College of Physicians and Surgeons of Canada Detweiler Travelling Fellowship. The funder/sponsor(s) had no role in design and conduct of the study, collection, analysis and interpretation of the data.

Author contributions

R.G.J., P.D.S., S.V., F.D.R., T.S. and B.H. participated in the study conception and design. Data acquisition, analysis and interpretation were performed by R.G.J., P.D.S., C.C.,

G.P.P., S.P., S.S., A.H., F.D.R., T.S. and B.H. Statistical analysis was performed by R.G.J., P.D.S. and B.H. The manuscript was drafted by R.G.J., P.D.S., F.D.R., T.S. and B.H. All authors approved the final version of the manuscript and agree to be accountable to all aspects of the work.

Competing interests

B.H. reports funding as a clinical trial investigator from Abbott, Boston Scientific and Edwards Lifesciences outside of the submitted work. The remaining authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-21220-5.

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Peer review information *Nature Communications* Ian White and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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Appendix B

RIPK1 expression associates with inflammation in early atherosclerosis in humans and can be therapeutically singled to reduce NF-kB activation and atherogenesis in mice

Citation information:

Karunakaran D, Nguyen MA, Geoffrion M, Vreeken D, Lister Z, Cheng HS, Otte N, Essebier P, Wyatt H, Kandiah JW, **Jung R**, Alenghat FJ, Mompeon A, Lee R, Pan C, Gordon E, Rasheed A, Lusis AJ, Liu P, Matic LP, Hedin U, Fish JE, Guo L, Kolodgie F, Virmani R, Rayner KJ. Therapeutic knockdown of RIPK1 gene expression reduces NF-κB inflammation in macrophages and prevents atherosclerotic lesion development. (**2021**). *Circulation*.

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RIPK1 Expression Associates With Inflammation in Early Atherosclerosis in Humans and Can Be Therapeutically Silenced to Reduce NF-κB Activation and Atherogenesis in Mice

Author: Denuja Karunakaran, My-Anh Nguyen, Michele Geoffrion, et al

Publication: Circulation

Publisher: Wolters Kluwer Health, Inc.

Date: Nov 23, 2020

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Circulation

ORIGINAL RESEARCH ARTICLE

RIPK1 Expression Associates With Inflammation in Early Atherosclerosis in Humans and Can Be Therapeutically Silenced to Reduce NF-κB Activation and Atherogenesis in Mice

BACKGROUND: Chronic activation of the innate immune system drives inflammation and contributes directly to atherosclerosis. We previously showed that macrophages in the atherogenic plaque undergo RIPK3 (receptor-interacting serine/threonine-protein kinase 3)-MLKL (mixed lineage kinase domain-like protein)–dependent programmed necroptosis in response to sterile ligands such as oxidized low-density lipoprotein and damage-associated molecular patterns and that necroptosis is active in advanced atherosclerotic plaques. Upstream of the RIPK3-MLKL necroptotic machinery lies RIPK1 (receptor-interacting serine/threonine-protein kinase 1), which acts as a master switch that controls whether the cell undergoes NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells)–dependent inflammation, caspase-dependent apoptosis, or necroptosis in response to extracellular stimuli. We therefore set out to investigate the role of RIPK1 in the development of atherosclerosis, which is driven largely by NF-κB–dependent inflammation at early stages. We hypothesize that, unlike RIPK3 and MLKL, RIPK1 primarily drives NF-κB–dependent inflammation in early atherogenic lesions, and knocking down RIPK1 will reduce inflammatory cell activation and protect against the progression of atherosclerosis.

METHODS: We examined expression of RIPK1 protein and mRNA in both human and mouse atherosclerotic lesions, and used loss-of-function approaches in vitro in macrophages and endothelial cells to measure inflammatory responses. We administered weekly injections of RIPK1 antisense oligonucleotides to *Apoe*–/– mice fed a cholesterol-rich (Western) diet for 8 weeks.

RESULTS: We find that RIPK1 expression is abundant in early-stage atherosclerotic lesions in both humans and mice. Treatment with RIPK1 antisense oligonucleotides led to a reduction in aortic sinus and en face lesion areas (47.2% or 58.8% decrease relative to control, $P<0.01$) and plasma inflammatory cytokines (IL-1α [interleukin 1α], IL-17A [interleukin 17A], $P<0.05$) in comparison with controls. *RIPK1* knockdown in macrophages decreased inflammatory genes (NF-κB, TNFα [tumor necrosis factor α], IL-1α) and in vivo lipopolysaccharide- and atherogenic diet–induced NF-κB activation. In endothelial cells, knockdown of *RIPK1* prevented NF-κB translocation to the nucleus in response to TNFα, where accordingly there was a reduction in gene expression of *IL1B*, *E-selectin*, and monocyte attachment.

CONCLUSIONS: We identify RIPK1 as a central driver of inflammation in atherosclerosis by its ability to activate the NF-κB pathway and promote inflammatory cytokine release. Given the high levels of RIPK1 expression in human atherosclerotic lesions, our study suggests RIPK1 as a future therapeutic target to reduce residual inflammation in patients at high risk of coronary artery disease.

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The full author list is available on page 175.

Key Words: atherosclerosis ■ inflammation ■ macrophages ■ NF-kappa B ■ RIPK1 protein, human

Sources of Funding, see page 176

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ORIGINAL RESEARCH ARTICLE

Clinical Perspective

What Is New?

- RIPK1 (receptor-interacting serine/threonine-protein kinase 1) acts as a proinflammatory hub in the vessel wall, with increased expression and coincident activation of NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells) in early atherosclerotic disease in humans.
- Reducing RIPK1 expression in mice prevents atherosclerosis development, in part, by reducing endothelial and macrophage activation of inflammatory pathways, including interleukin 1α and interleukin 1β.

What Are the Clinical Implications?

- Inflammation as a therapeutic target for atherosclerotic vascular disease was recently validated by the CANTOS trial (Cardiovascular Risk Reduction Study [Reduction in Recurrent Major CV Disease Events]), and we identify herein that the therapeutic silencing of RIPK1 acts as a parallel but alternative strategy to dampen inflammation in the vessel wall.
- Antisense oligonucleotides can effectively decrease RIPK1 expression and activation without the unwanted spontaneous activation of inflammatory cell death that accompanies complete loss of RIPK1, making this approach readily translatable to human disease.

Atherosclerosis begins as an innate immune response to modified cholesterol-rich lipoproteins trapped in the subendothelial space of the vessel wall, recruiting inflammatory monocytes from the circulation.[1,2] These monocytes differentiate into macrophages that avidly engulf modified lipids (eg, oxidized low-density lipoprotein) through scavenger receptors (eg, CD36, SR-A) to become foam cells. Although these foam cells initially play a protective role in mediating the efflux of cholesterol from the lesions, they also engage the NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells) pathway to transcriptionally activate the production of proinflammatory cytokines (eg, TNFα [tumor necrosis factor α], GM-CSF [granulocyte-macrophage colony-stimulating factor], IL-1α/β [interleukin 1α/β]), and chemokines to sustain the local inflammatory environment, resulting in additional monocyte recruitment.[3] Furthermore, double-hit engagement by modified lipoproteins and other sterile inflammatory cues (eg, ATP) can trigger NF-κB–regulated NLRP3 (NLR family pyrin domain containing 3) inflammasome-dependent IL-1β (interleukin 1β)[4] or inflammasome-independent and oxidative stress–dependent IL-1α (interleukin 1α) secretion.[5] As foam cells become overwhelmed with lipid, they can undergo cell death. To date, several types of cell death pathways have been identified to occur with atherosclerotic lesions: noninflammatory apoptosis, proinflammatory necroptosis, and secondary necrosis.[6–10] The ineffective clearance of these dying macrophages by efferocytosis adds to the lesional burden, forming a large necrotic core that destabilizes the atherosclerotic plaque.[11,12] In the past 3 decades, low-density lipoprotein (LDL)–lowering therapies have emerged as the leading therapeutic strategy to successfully prevent or treat atherosclerosis. Although such therapies are highly effective, they reduce atherosclerotic disease incidence by only 50% to 60%, leaving considerable residual disease burden.[13] Moreover, with the recent failures of high-density lipoprotein–raising therapies as an alternative therapeutic approach,[14] there is renewed focus in finding novel therapeutic targets that act in parallel with cholesterol-lowering drugs to eliminate residual inflammation within lesions. The outcomes of 2 large clinical trials targeting inflammation in coronary artery disease, CANTOS (Cardiovascular Risk Reduction Study [Reduction in Recurrent Major CV Disease Events]),[15] which used an IL-1β monoclonal antibody, and COLCOT (Colchicine Cardiovascular Outcomes Trial),[16] which used low-dose anti-inflammatory colchicine; both demonstrated reduced cardiovascular events in high-risk patients with myocardial infarction. These studies provide proof-of-concept and pave the way to an era of exciting new possibilities for anti-inflammatory therapies to treat cardiovascular diseases.

RIPK1 (receptor-interacting serine/threonine-protein kinase 1) and RIPK3 (receptor-interacting serine/threonine-protein kinase 3) belong to a family of serine-threonine kinases that execute innate immune signaling and cell stress pathways.[17] Activation of TNFR1 (TNF receptor 1) recruits and assembles complex I, consisting of RIPK1, adaptor proteins (eg, TRADD [TNF receptor–associated death domain] and TRAF2 [TNF receptor–associated factor 2]), E3 ubiquitin ligases, and cellular inhibitor of apoptosis at the cytoplasmic tail of the receptor. Cell survival and death pathways are carefully orchestrated by reciprocal phosphorylation and ubiquitination of RIPK1. When RIPK1 is modified by ubiquitination, it forms a scaffold for the stabilization of IKKα/IKKβ (IκB kinase α/IκB kinase β), degradation of IκBα, release of NF-κB, and activation of inflammation and prosurvival genes.[18] When RIPK1 is deubiquitinated, this promotes RIPK1 activation of apoptosis and necroptosis.[19] To activate necroptosis, RIPK1 interacts with and activates RIPK3, which subsequently recruits and phosphorylates the pseudokinase MLKL (mixed lineage kinase domain-like protein). MLKL oligomerizes and promotes the rupture of the plasma membrane, causing release of ATP, mitochondria, and other damage-associated molecular patterns and a cell that shares morphological features with necrosis. The balance of RIPK1 signaling in response to inflammatory challenges

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is necessary to control cell survival, death, and mounting an inflammatory response.[20,21] However, the precise ways in which RIPK1 controls these pathways are still being elucidated. We and others have previously shown that therapeutic targeting of RIPK3 phosphorylation or its genetic deletion markedly reduces macrophage necroptosis and the development of the necrotic core within the atherosclerotic plaque, promoting lesional stability in advanced atherosclerosis.[22,23] Deletion of RIPK3 only reduced atherosclerotic lesion area after long-term Western diet feeding, but it did not protect against early atherosclerosis, suggesting that necroptosis may not contribute to lesion initiation.[23] Unlike RIPK3, the role for its upstream regulator RIPK1 in atherosclerosis has not been studied.

Here, for the first time, we investigate the role of RIPK1, a coordinator of NF-κB inflammation and cell death, in atherosclerosis. We find that RIPK1 expression is highly expressed in early atherosclerotic lesions in humans and mice. In vitro, both basal and TNFα-stimulated NF-κB activity and resultant inflammatory gene expression are reduced in macrophages and endothelial cells when RIPK1 is silenced. In vivo, therapeutic administration of RIPK1 antisense oligonucleotides (ASOs) markedly reduces atherosclerotic lesion size and macrophage content. Together, these findings suggest that RIPK1 drives inflammation in early atherosclerosis, and targeting RIPK1 provides a novel preventative therapeutic strategy to treat atherosclerosis.

METHODS

The data, methods, and study samples will be made available to other researchers for purposes of reproducing the results or replicating the procedures according to the American Heart Association journals' implementation of the Transparency and Openness Promotion Guidelines.

Reagents

Human LDL was purchased from Alfa Aesar and oxidized according to previous methods (Cu_2SO_4 oxidation 6 hours).[24] M-CSF (macrophage colony-stimulating factor) and TNFα were obtained from BioLegend. Mouse IL-1α and IL-1β DuoSet ELISA kits were from R&D Systems. zVAD.fmk (zVAD) and lipopolysaccharide (LPS) were purchased from BioVision and Sigma-Aldrich, respectively.

Human Atherosclerosis

All human studies were done with approval from institutional review committees and subjects gave informed consent. To determine the association between RIPK1 mRNA expression levels and other markers of interest in human carotid plaques, Pearson correlations were calculated from the microarray data set belonging to BiKE (Biobank of Karolinska Endarterectomies) (GEO [Gene Expression Omnibus] database access: GSE21545), using GraphPad Prism 6 software. The data set contains 127 plaques from patients who underwent surgery for high-grade (>50% NASCET [North American Symptomatic Carotid Endarterectomy Trial]) carotid stenosis at the Department of Vascular Surgery, Karolinska University Hospital, Stockholm, Sweden.[25] The BiKE study cohort demographics, details of sample collection and processing, and transcriptomic analyses by microarrays were previously described in detail.[26] Human carotid endarterectomy sample data were also obtained from an independent data set (GEO access: GSE43292) from the NCBI GEO data repository,[27] where RIPK1 and CD68 expression values were obtained by comparing severe plaque (stage IV and V) to adjacent areas with early disease only (no macroscopic disease, stage I and II).[28]

For immunofluorescence analysis, human coronary artery samples were obtained from the CVPath Institute Sudden Cardiac Death registry as previously described.[29] Comprehensive analysis of coronary artery histology was performed for each subject, and the samples were categorized as control lesions with pathological intimal thickening, early and late fibroatheromas by examining hematoxylin and eosin staining. Formalin-fixed and paraffin-embedded coronary artery blocks were sectioned at 5 mm thickness. These sections were stained with RIPK1 polyclonal antibody (No. PA5-2811, ThermoFisher, 1:100) and then with secondary goat anti-rabbit antibody (No. AA11037, ThermoFisher, 1:500).

Experimental Animals

Male and female C57Bl/6 wild-type and $ApoE^{-/-}$ mice were purchased from Charles River or Animal Resources Center (Western Australia). $RIPK1^{KD}$ ($RIPK1^{K45A}$) mice were a generous gift from Dr Peter Gough[30] (GlaxoSmithKline) and $ApoE^{-/-}$/NF-κB transgenic mice were provided by Dr Mark Feinberg (Brigham & Women's Hospital, Boston).[31] All animal studies were permitted by the University of Ottawa Animal Care and Use Committee in accordance with the international standards established by the Canadian Council on Animal Care or The University of Queensland Animal Ethics Committee (IMB/337/19) in Australia.

Gene Expression Analysis in Mouse Aorta

Gene expression analysis in mouse aorta from the Hybrid Mouse Diversity Panel was performed as described previously.[32] In brief, the Hybrid Mouse Diversity Panel consists of ≈100 unique inbred mouse strains that have been phenotyped extensively for cardiometabolic traits that can be correlated to gene expression data. We analyzed aortic gene expression from mice across the 100 strains fed a 1% cholesterol-enriched diet, as described previously.[33] All correlations were calculated using biweight midcorrelation (implemented in the bicor) function from the WGCNA R package), which is analogous to Pearson correlation but is more robust to outliers because the underlying calculations are median based rather than mean based and are expressed as correlation coefficient (R) and P value.

To examine gene expression in the lesser and greater curvature, *Ldlr*–/– mice were fed a Western diet for 4 weeks and the ascending aorta was dissected, and cells were extracted through a DNase I and Liberase enzyme treatment as previously described.[34] Enzymes were then quenched and washed,

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and intimal cells were gently scraped into RNA extraction buffer (MicroRNAeasy RNA isolation kit, Qiagen). Isolated RNA was reversed transcribed using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) and quantitative reverse transcription polymerase chain reaction was performed using SYBR Green I Master mix (Roche) according to the manufacturer's instructions. Gene expression was normalized to hypoxanthine-guanine phosphoribosyltransferase using the Delta-Delta Ct method.

Macrophage Isolation

Bone marrow cells were isolated from femurs of adult wild-type mice and differentiated into macrophages (bone marrow–derived macrophages) using DMEM supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and either 20% L929 conditioned medium or 20 ng/mL M-CSF as previously described.[10] To polarize macrophages to M1 or M2, cells were treated with 500 ng/mL LPS and 100ng/mL interferon-γ, or 10 ng/mL IL-4 (interleukin 4) for 24 hours. For oxidized low-density lipoprotein (oxLDL) treatment, cells were treated with 100 µg/mL oxLDL with or without 50 µmol/L zvad.fmk.

Wild-type, adult mice (8 weeks old) were subjected to an intraperitoneal injection of 1 mL of 3% or 10% thioglycollate. For in vivo transfection of ASOs, 3 days after thioglycollate injection, the mice are subjected to another intraperitoneal injection of 50 mg/kg either control (CCTTCCCTGAAGGTTCCTCC a random nontargeting sequence) or RIPK1 (TCAGCCACTTCTGAAGCATT) ASOs from Ionis Pharmaceuticals. On day 4 after thioglycollate, peritoneal macrophages were isolated by peritoneal lavage with 2×5 mL sterile phosphate-buffered saline as described.[35] Cells were centrifuged, resuspended in DMEM supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and seeded at 4×10^6 cells/well in a 6-well plate, 1×10^6 cells/well in a 24-well plate, and 0.2×10^6 cells/well in a 96-well plate and incubated at 37°C for 24 hours. Cells were treated with 100 µg/mL oxLDL, 50 ng/mL TNFα, 50 µmol/L zvad.fmk, as described in the figure legends. For cytokine ELISAs, cells were primed with 200 ng/mL LPS and then treated with 50 µg/mL oxLDL for 18 hours.

Endothelial Cells

Primary human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords obtained at the Leiden University Medical Center after written informed consent and ensuring that collection and processing of the umbilical cord was performed anonymously. The umbilical vein was flushed with phosphate-buffered saline, using glass cannulas, to remove all remaining blood. Endothelial cells were detached by infusion of the vein with Trypsin/EDTA (1×) (Lonza, BE02-007E) solution and incubation at 37°C for 15 minutes. After incubation, the cell suspension was collected and taken up in endothelial cell growth medium (EGM2 medium, Promocell, C-22010) with 1% antibiotics. After flushing the umbilical vein once more with phosphate-buffered saline, to ensure all detached cells are collected, cells were pelleted by centrifugation at 1200 rpm for 7 minutes. The cell pellet was dissolved in fresh EGM2 medium and cells were cultured on gelatin (1%)-coated surfaces. For TNFα stimulation, HUVECs were incubated with 2 ng/mL TNFα for 24 hours, unless indicated otherwise. To achieve a knockdown of RIPK1, HUVECs were transduced with lentiviral particles encoding short hairpin RNA targeting RIPK1 (MISSION library Sigma-Aldrich, TRCN0000000705, TRCN0000000706, TRCN0000000708, TRCN0000200006) or a scrambled control short hairpin RNA (mock). Selection of transduced cells was achieved using puromycin (2 µg/mL).

Monocyte Adhesion Assay

THP1 cells (ATCC, TIB-202) were cultured in RPMI 1640 medium (Gibco, 22409) supplemented with 10% fetal calf serum, 1% L-glutamine, 1% antibiotics (penicillin/streptomycin, Gibco, 15070063), and 25 nmol/L β-mercaptoethanol. THP1 cells were labeled with 5 µg/mL Calcein AM (Molecular Probes Life Technologies, C3100MP) and incubated on top of a monolayer of HUVECs, pretreated with 2 ng/mL TNFα for 24 hours, for 30 minutes at 37°C. Nonadherent cells were washed away by multiple washing steps with phosphate-buffered saline after which the cells were lysed in Triton-X 0.5% for 10 minutes. Fluorescence was measured at excitation wavelength 485 nm and emission wavelength 514 nm. Each condition was performed in triplicate.

Gene Expression

Treated cells were lysed in TRIzol reagent (Invitrogen) according to the manufacturer's recommendations, and complementary DNA was synthesized using iScript Reverse Transcription kit (Bio-Rad). Quantitative polymerase chain reaction was performed in triplicate using TaqMan Gene Expression Assay and mRNA level was normalized to either β-2-microglobulin or hypoxanthine-guanine phosphoribosyltransferase. For HUVECs, total RNA was reverse transcribed using M-MLV Reverse Transcriptase Kit (Promega, M1701). Reverse transcription polymerase chain reaction analysis was conducted using SYBR Select Master Mix (Applied Biosystems, 4472908). mRNA expression was normalized to expression of GAPDH.

Western Blot

Peritoneal macrophages (1.8×10^6 per well) were lysed directly in 2× SDS sample buffer (Bio-Rad) and subjected to SDS-PAGE and Western blot analysis with RIPK1 antibody (1:1000, No. 610458, BD Transduction Laboratories).

Cell Viability Assays

Lactate dehydrogenase release into the medium was detected as a kinetic assay by measuring absorbance at 340 nm for 1-minute intervals for 10 minutes as previously described.[10] The slope of the curve was expressed as a fold change relative to control.

NF-κB Luciferase Assay

Adult ApoE$^{-/-}$/NF-κB-reporter mice were fed a chow or Western diet (0.2% cholesterol; Harlen Teklad) for 4 weeks. These mice were then subjected to an intraperitoneal injection of thioglycollate, followed by another intraperitoneal injection of control or RIPK1 ASO for 36 hours before isolation of macrophages. The NF-κB promoter activity was measured as

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luciferase signal detected by using the Dual-Luciferase assay (Promega) according to the manufacturer's instructions. All assays were performed from either 3 (Western diet–fed) or 6 (LPS-treated) mice per group in 4 technical replicates.

Immunofluorescence of HUVECs

For immunofluorescence staining, HUVECs were cultured on µ-Slide 8 well (Ibidi, C80826), stimulated with or without 2 ng/mL TNFα for 24 hours, and fixed with 4% formaldehyde (v/v in Hank's balanced salt solution [HBSS] supplemented with calcium and magnesium [HBSS+]), Gibco) for 10 minutes. Cells were then permeabilized with 0.1% Triton X-100 and blocked with 5% BSA (wt/wt in HBSS+) for 30 minutes. The primary antibody was prepared in 0.5% BSA (w/wv in HBSS+): intercellular adhesion molecule 1 mouse anti-human antibody (Santa Cruz Biotechnology, SC-107, 1:50), NF-κB p65 rabbit anti-human antibody (Cell Signaling Technology, 8242, 1:400). Incubation of the primary antibody was done at room temperature for 1 hour. The secondary antibody was also prepared in 0.5% BSA (goat anti-mouse IgG alexa 488, goat anti-rabbit alexa 647, Invitrogen, 1:500), together with Rhodamine Phalloidin (Invitrogen, 1:200) and Hoechst 33528 (Invitrogen, H3569, 1:2000) to stain F-actin and cell nuclei. Fluorescence images were captured by using the ImageXpress (Molecular Devices) and analyzed using ImageJ software.

Atherosclerosis Studies

Eight-week-old female and male ApoE$^{-/-}$ mice (9–12/group) were fed an adjusted calories Western diet (21% fat, 0.2% cholesterol; Harlan Teklad) for 8 weeks and were randomly assigned to simultaneously receive weekly subcutaneous injections of 100 mg/kg control ASO (CCTTCCCTGAAGGTTCCTCC) or RIPK1 ASO (TCAGCCACTTCTGAAGCATT) or for indicated experiments, RIPK1 ASO-B (CTCCATGTACTCCATCACCA). After 8 weeks, mice were euthanized; perfused with saline; the aortic root, ascending and descending aorta, and liver were collected as previously described.[10,35,36] In brief, aortic sinuses were embedded in optimum cutting temperature compound medium, frozen, sectioned (10 µm), and stained with hematoxylin and eosin for quantifications, where a minimum of 8 to 10 sections per animal were measured across the length of the entire aortic root. For en face aortic lesion quantifications, dissected aortas (down to the femoral bifurcation) were sliced ventrally and imaged; the lesion area was quantified by a nonblinded user using ImageJ. To visualize macrophages, smooth muscle cells (SMCs), IL-1α immunofluorescence was performed using Mac-2 antibody (Cedarlane, CL8942AP) followed by Alexa488-conjugated secondary antibody, SMCα (monoclonal antibody conjugated to alkaline phosphatase, Sigma, A5691) or IL-1α (Abcam ab7632) followed by Alexa594-conjugated secondary antibody. Sections were imaged using the Leica ScanScope. Fluorescent positive areas were quantified using ImageJ. Serum cytokines were analyzed using Bioplex Pro Mouse Cytokine Assay (no. M60009RDPD; Bio-Rad) according to the manufacturer's instructions.

Statistical Analysis

Data shown are either a representative mean±SD experiment of at least 3 experiments or mean±SEM of experiments performed in triplicate, as indicated in the corresponding figure legend. For comparison between 2 groups, Student *t* tests (unpaired) were used, unless otherwise stated, and *P*- values are indicated in the figure legends. For comparison between groups, 1- or 2-way ANOVA ($P \leq 0.05$) with multiple comparisons tests was performed using Graphpad Prism.

RESULTS

RIPK1 Expression Is Increased in Early-Stage Atherosclerotic Lesions

To begin to understand how RIPK1 might be contributing to lesion development, we examined its expression in lesions with different degrees of atherosclerotic disease burden. In human coronary arteries with disease classified as pathological intimal thickening or in those with larger early fibroatheromas, RIPK1 protein was detected within the intimal space (Figure 1A; red). Double immunostaining in these arteries indicates that RIPK1 is expressed both in macrophages (marked by CD68) and endothelial cells (marked by vWF; Figure 1B). In endarterectomy samples from individuals with carotid atherosclerosis, the mRNA expression of RIPK1 correlated strongly with the expression of genes in the NF-κB pathway *IKKA*, *IKKB*, *NFKB1*, and the downstream effector *IL-1A* (Figure 1C, all $P<0.0001$). In another gene expression data set from human carotid endarterectomies (GEO data set GSE43292), we find that the expression of RIPK1 on a per macrophage basis (ie, per copy of *CD68* gene expression) is higher in lesions with early-stage disease (stages I and II) than in adjacent regions with more severe plaque (stages IV and V) (Figure 1D). To further evaluate *Ripk1* expression in mouse atherosclerosis, we compared gene expression across 100 strains of outbred mice from the Hybrid Mouse Diversity Panel and find that expression of *Ripk1* in the aorta strongly correlates with expression of *Ikbkb*, an activator of the NF-κB pathway (Figure 1E). In mice with early-stage atherosclerotic lesions (*Ldlr*–/– mice fed a high-fat diet for 4 weeks[34]), we find that gene expression of both *Ripk1* and markers of inflammation (*Tnfa*, *Ccl2*, *Il1b*) are higher in the athero-susceptible, lesser curvature of the aorta relative to the athero-resistant greater curvature aortic region (Figure 1F). Together, these data demonstrate that RIPK1 is highly expressed in early-stage atherosclerotic lesions in both humans and mice and correlates with indices of inflammation in the plaque.

Loss of *Ripk1* Reduces Macrophage Inflammation in Vitro and NF-κB Activation In Vivo

We next set out to assess the expression profile of RIPK1 in inflammatory macrophages in vitro. We measured

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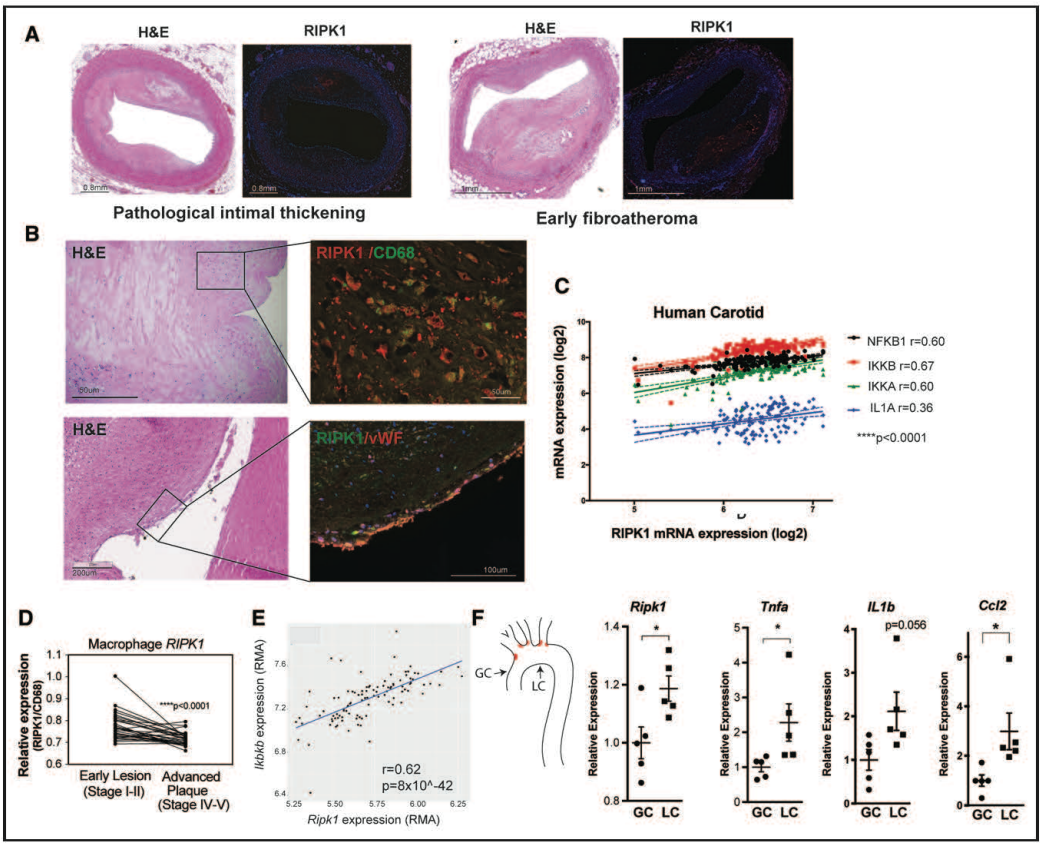


Figure 1. RIPK1 is expressed in human and mouse atherosderotic lesions.
A and **B**, Human coronary arteries with atherosclerotic lesions classified as either pathological intimal thickening or early fibroatheroma. H&E staining and RIPK1 staining (red) indicate that RIPK1 is expressed in both stages of lesion development (**A**) in both CD68+ (macrophages) and vWF+ cells (endothelial cells) (**B**). **C**, Correlations of the RIPK1 transcript with expression of *NFKB*, *IKKA*, *IKK8*, and *IL1A* in human carotid endarterectomies (n=127). ****P<0.0001 by Pearson correlation. **D**, Analysis of *RIPK1* gene expression data from human carotid artery lesions classified as early (stage I–III) or advanced (stage IV and V) from Gene Expression Omnibus data set GSE43292 normalized to the expression of CD68. ****P<0.0001 by paired *t* test. **E**, *Ripk1* mRNA expression in the aorta of mice from the Hybrid Mouse Diversity Panel (100 inbred strains of mice) and the correlation with *Ikbkb* mRNA in the aorta. Correlation coefficient (*R*) and *P* value were calculated as described in the Methods. **F**, Mouse *Ripk1*, *Tnfa*, *Il1b*, and *Ccl2* gene expression in athero-prone (LC) and athero-protective (GC) regions of atherosclerotic lesions isolated from *Ldlr*$^{-/-}$ mice fed a high-fat diet for 4 weeks. *P≤0.05, Student *t* test. GC indicates greater curvature; H&E, hematoxylin and eosin; LC, lesser curvature; RIPK1, receptor-interacting serine/threonine-protein kinase 1; RMA, robust multi-array average; and vWF, von Willebrand factor.

Ripk1 gene expression in bone marrow–derived macrophages polarized to either a proinflammatory M1 or anti-inflammatory M2 macrophages, and we observed a 3.2-fold increase in *Ripk1* gene expression in M1 macrophages relative to nontreated M0-macrophages, whereas *Ripk1* levels in M2 macrophages were unchanged (Figure 2A).We had previously shown that oxLDL induces the expression of necroptotic genes, *Ripk3* and *Mlkl*. Here, we similarly find that oxLDL and oxLDL+zVAD treatment of bone marrow–derived macrophages resulted in a 1.4- and 1.8-fold increase in *Ripk1* gene expression, respectively (Figure 2B).

Using ASOs to knockdown expression of *Ripk1*, we evaluated the activation of inflammation in vivo in thioglycollate-elicited macrophages in the peritoneal cavity. Intraperitoneal injection of RIPK1 antisense decreased both *Ripk1* gene and protein expression relative to control, as expected (Figure 2C). In macrophages treated with RIPK1 antisense, we also found a reduction in baseline expression of inflammatory transcripts *Nfkb1* and *Tnfa* (67.46%±21.55% and 58.85%±24.75%) and a decrease in inflammasome-related genes *Casp1*, *Nlrp3*, *Il1a*, and *Il1b* (55.45%±31.03%, 61.92%±27.87%, 44.07%±7.52%, 55.22%±6.99%, respectively), as well (Figure 2D). To investigate the role of RIPK1 in driving TNFα- or oxLDL-induced inflammation, we isolated peritoneal macrophages after RIPK1 silencing

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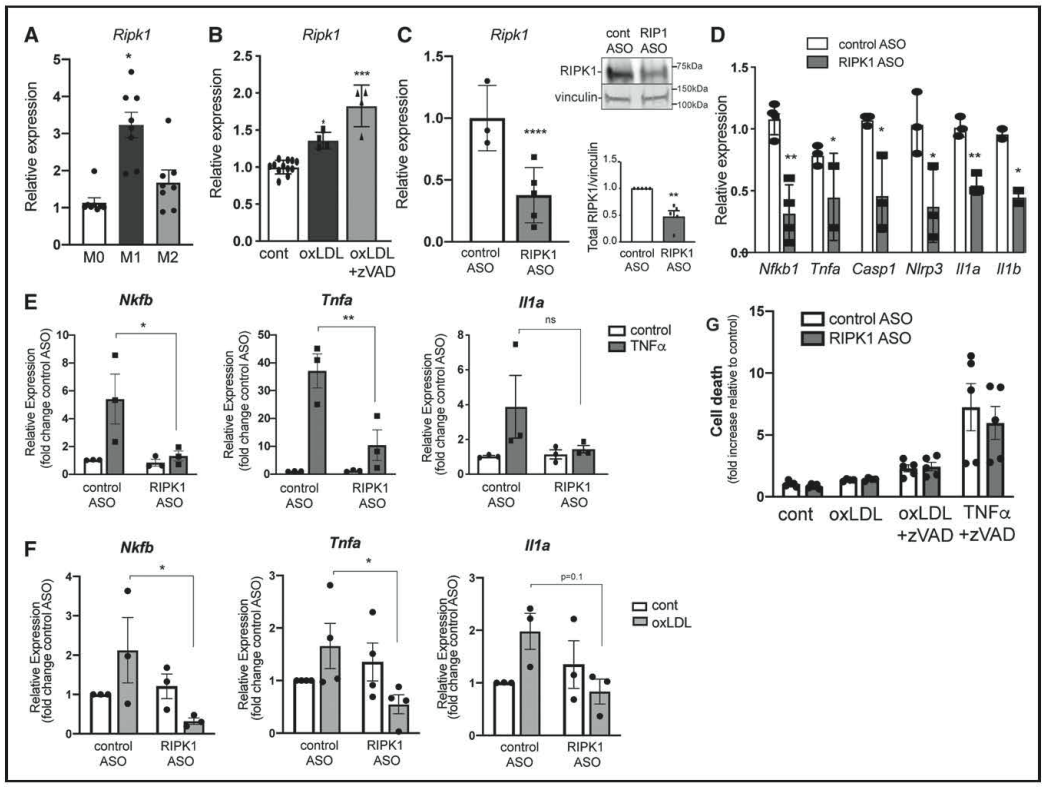


Figure 2. *Ripk1* expression is induced in inflammatory macrophages and knockdown of *Ripk1* reduces inflammatory gene activation.
A, *Ripk1* gene expression in bone marrow–derived macrophages polarized to either a proinflammatory M1 (lipopolysaccharide and interferon-γ treated) or anti-inflammatory M2 (IL-4 treated) macrophages. **B**, Treatment of bone marrow–derived macrophages with 100 μg/mL oxLDL, with or without 50 μmol/L zVAD before RNA isolation and analysis of *Ripk1* gene expression relative to housekeeping genes. For **A** and **B**, **P*<0.01 and ****P*<0.001 by 1-way ANOVA and Tukey multiple comparisons test. **C** and **D**, Intraperitoneal injection of 50 mg/kg control ASO or RIPK1 ASO before peritoneal macrophage isolation and gene expression analysis of *Ripk1* (**C**) and inflammatory genes *Nfkb*, *Tnfa*, *Casp1*, *Nlrp3*, *Il1a*, and *Il1b* (**D**). **C** and **D**, **P* ≤ 0.05, *****P*<0.0001 by unpaired Student *t* test. **E** and **F**, Peritoneal macrophages transfected with control ASO or RIPK1 ASO and treated with 50 ng/mL TNFα or 100 μg/mL oxLDL before gene expression analysis of *Nfkb*, *Il1a*, and *Tnfa*. **G**, Cell viability of peritoneal macrophages transfected with control ASO or RIPK1 ASO and treated with oxLDL, oxLDL+zVAD, TNFα+zVAD, lipopolysaccharide+zVAD. For **E** through **G**, **P*<0.05, ***P*<0.01, and ****P*<0.001 by 2-way ANOVA and Sidak multiple comparisons test. ASO indicates antisense oligonucleotide; cont, control; ns, not significant; oxLDL, oxidized low-density lipoprotein; RIPK1, receptor-interacting serine/threonine-protein kinase 1; TNFα, tumor necrosis factor α; and zVAD, pan-caspase inhibitor z-Vad-fmk.

and treated the cells with TNFα or oxLDL before analyzing inflammatory gene expression. The TNFα– and oxLDL-stimulated expression of *Nfkb1*, *Il1a*, and *Tnfa* was reduced in peritoneal macrophages transfected with RIPK1 antisense (Figure 2E and 2F). We and others have shown that atherogenic ligands induce RIPK3-dependent necroptosis,[10,23,37] and inactivation of RIPK1 kinase activity has been shown previously to reduce necroptotic cell death.[30,38] We therefore tested whether reducing RIPK1 gene expression also affected the activation of necroptosis. We observed that knocking down RIPK1 did not affect necroptotic ligand (oxLDL, oxLDL+zVAD, TNFα+zVAD, LPS+zVAD)–induced cell death in peritoneal macrophages unlike what is observed in peritoneal macrophages from RIPK1 kinase-dead (RIPK1^{K45A}) mice (Figure 2G and Figure IA and IB in the Data Supplement). Together, these data suggest that knocking down RIPK1 impairs the ability of macrophages to promote NF-κB–mediated inflammation while maintaining their capacity for necroptotic cell death.

To test whether RIPK1 directly targets NF-κB activity in atherogenic macrophages, we used transgenic *Apoe*–/– mice expressing the NF-κB promoter upstream of a green fluorescent protein/luciferase fusion reporter gene.[31,39] Macrophages were elicited into the peritoneal cavity of ApoE–/–/NF-κB reporter mice, injected with control or RIPK1 antisense, followed by a single LPS injection to induce an inflammatory response. As expected, LPS treatment significantly increased NF-κB promoter activity in in vivo macrophages (9-fold), and this was inhibited in RIPK1-silenced macrophages

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(Figure 3A). We were then interested in whether atherogenic inflammatory activation of NF-κB was also inhibited by RIPK1 knockdown; therefore, we fed *Apoe*−/−/NF-κB reporter mice a Western diet (0.2% cholesterol) for 4 weeks to induce hypercholesterolemia and foam cell formation in peritoneal macrophages before delivery of control or RIPK1 ASOs. Peritoneal macrophages isolated from Western diet–fed mice had a 1.7-fold increase in NF-κB activity in comparison with chow diet–fed mice (Figure 3B).RIPK1 antisense-transfected in vivo peritoneal macrophages isolated from both chow-fed and Western diet–fed mice had a marked reduction in NF-κB luciferase activity (Figure 3B), suggesting that both basal inflammatory and diet-induced inflammatory responses were attenuated by RIPK1 knockdown. Recent studies have shown that NF-κB activation induces gene regulation that drives NLRP3 inflammasome–dependent IL-1β and –independent IL-1α secretion from macrophages.[4] To investigate the role of RIPK1 in regulating macrophage IL-1 secretion, peritoneal macrophages transfected with control or RIPK1 antisense were primed with either LPS or TNFα before stimulation with oxLDL. In comparison with control cells, RIPK1 silencing in macrophages led to a reduction in secreted IL-1α in both LPS- and TNFα-primed macrophages (by ≈40% and 56%, respectively; Figure 3C, Figure IIA in the Data Supplement). There was also decreased IL-1β secretion in macrophages with RIPK1 antisense in LPS+oxLDL–stimulated cells (Figure 3D). IL-1β was not detected in TNFα+oxLDL–treated supernatants (data not shown). Together, these data suggest that RIPK1 expression downstream of atherogenic and proinflammatory stimuli drives NF-κB–dependent proinflammatory IL-1α and IL-1β secretion from inflammatory macrophages.

Knockdown of Endothelial RIPK1 Ameliorates TNFα-Induced Inflammation

NF-κB activation in endothelial cells (ECs) is critical for the initiation of the inflammatory response and induces the expression of adhesion molecules and inflammatory cytokines, among others. Because we observed a significant downregulation of NF-κB on reduction of RIPK1 in macrophages, we sought to examine the impact of *RIPK1* silencing on the endothelial inflammatory response. Treatment of HUVECs with TNFα for 24 hours induced the expression of *IL1B* in control ECs, but this response was reduced ≈85% when *RIPK1* was silenced (Figure 4A). A similar response was observed for E-selectin

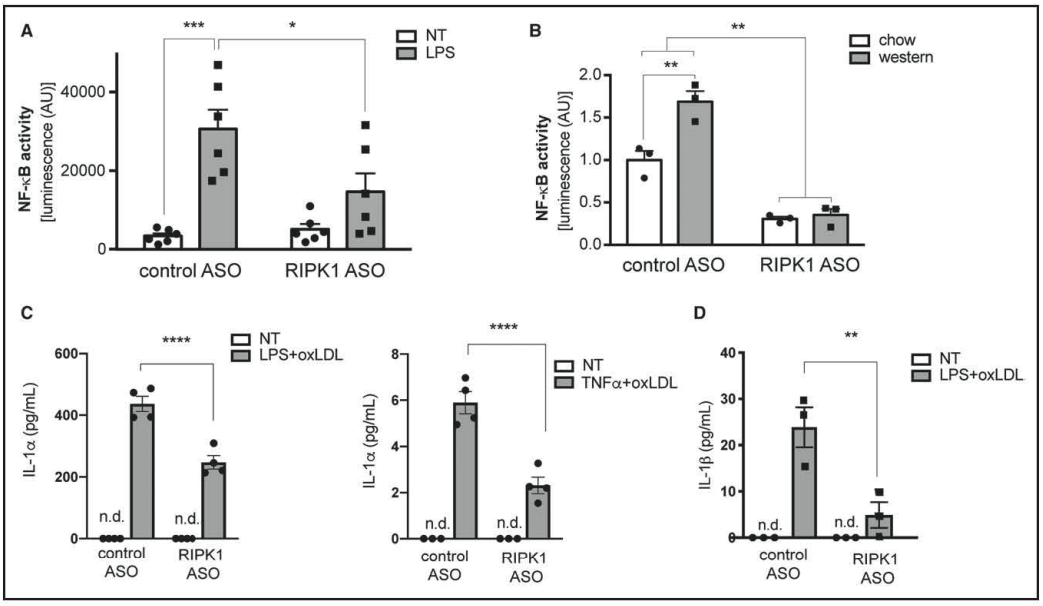


Figure 3. RIPK1 ASO markedly inhibits NF-κB activation in response to inflammatory stimuli in vivo.
A, Peritoneal macrophages were elicited in male and female ApoE (apolipoprotein E)-NF-κB reporter mice (n=6/group) with intraperitoneal injection of thioglycollate and, at day 3, the mice were administered 50 mg/kg control or RIPK1 ASO intraperitoneal injection. On day 5, the mice were subjected to intraperitoneal injection of 200 ng of LPS for 1.5 hours before the isolation of macrophages. **B**, Eight-week-old male mice (n=3/group) were subjected to a Western diet for 4 weeks before eliciting peritoneal macrophages and administration of RIPK1 ASOs as described in **A**. **C** and **D**, Wild-type peritoneal macrophages treated with 50 mg/kg control or RIPK1 ASO were primed with 200 ng/mL LPS or 50 ng/mL TNFα and treated with 50 μg/mL oxLDL for 18 hours in vitro. Secreted IL-1α (**C**) and IL-1β (**D**) were measured using ELISA. *P<0.05, **P<0.01, and ****P<0.001 by 2-way ANOVA and Sidak multiple comparisons test. ASO indicates antisense oligonucleotide; IL, interleukin; LPS, lipopolysaccharide; n.d., not detected; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; NT, no treatment; oxLDL, oxidized low-density lipoprotein; RIPK1, receptor-interacting serine/threonine-protein kinase 1; and TNFα, tumor necrosis factor α.

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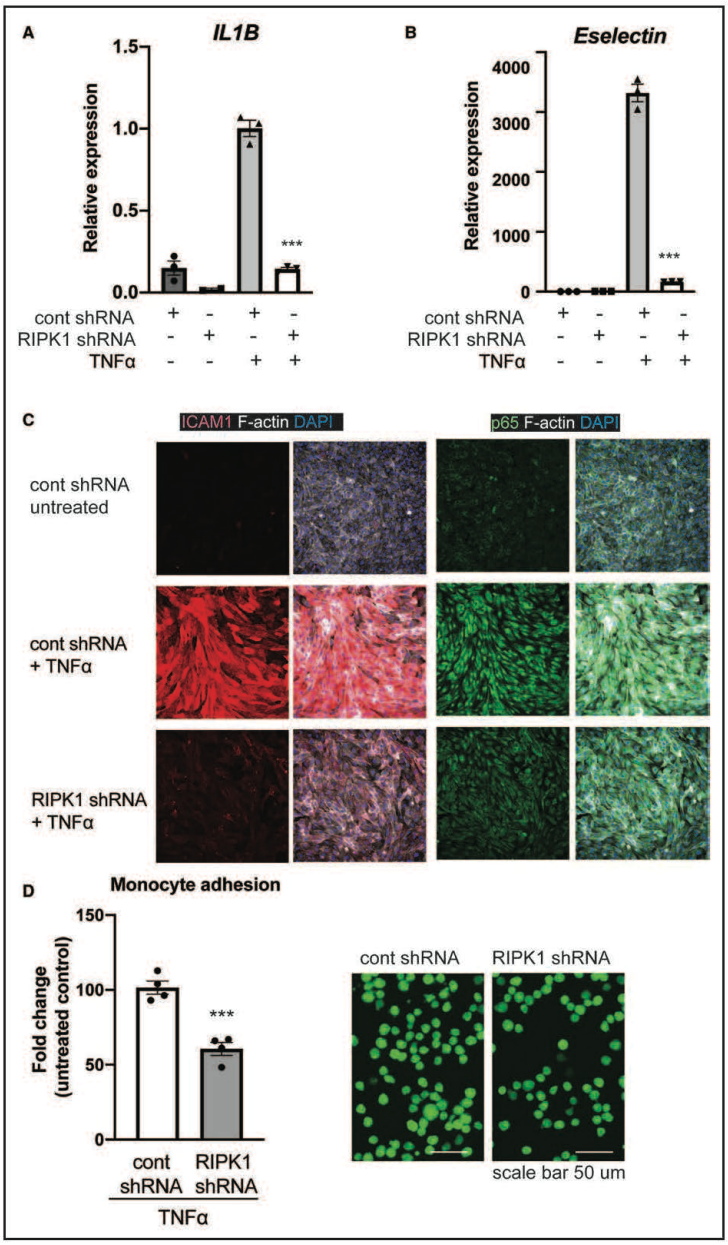


Figure 4. RIPK1 knockdown in endothelial cells reduces inflammatory gene expression and monocyte adhesion.
A through **C**, Human umbilical vein endothelial cells were transduced with lentiviral shRNA against RIPK1 (or mock control) and treated with TNFα for 24 hours before gene expression analysis (**A** and **B**) and immunofluorescence of ICAM-1 (red), p65 NF-κB subunit (green), F-actin (white), and DAPI (blue) (**C**); n=3 per treatment, experiments performed in triplicate; ***P<0.001 versus cont shRNA through 1-way ANOVA with the Tukey multiple comparisons test. **D**, THP-1 monocytes were labeled with calcein fluorescent dye (green) and incubated on top of a monolayer of human umbilical vein endothelial cells (transduced and treated TNFα for 24 hours as mentioned) for 30 minutes at 37°C. Cells were washed extensively before visualization by microscopy and quantification by plate reader. ***P<0.001 versus cont shRNA by the Student t test. Cont indicates control; DAPI, 4′,6-diamidino-2-phenylindole; ICAM-1, intercellular adhesion molecule 1; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; RIPK1, receptor-interacting serine/threonine-protein kinase 1; shRNA, short hairpin RNA; and TNFα, tumor necrosis factor α.

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(*SELE*), where *RIPK1* knockdown reduced TNFα-induced E-selectin expression by ≈95% (Figure 4B). Both the expression of intercellular adhesion molecule 1 and the nuclear translocation of the p65 subunit of NF-κB were induced on TNFα treatment, and *RIPK1* knockdown blunted these responses (Figure 4C). To determine if knocking down *RIPK1* functionally altered the EC inflammatory phenotype, monocytes were incubated with ECs after stimulation with TNFα. In comparison with controls, silencing of *RIPK1* led to a decrease in monocyte adhesion to the endothelial monolayer (Figure 4D). Because complete loss of RIPK1 can lead to spontaneous cell death, we tested whether ECs with RIPK1 knockdown were more susceptible to cell death in response to TNFα. We saw no difference in cell death between control and RIPK1-silenced cells (Figure IIB in the Data Supplement). Together, the data suggest that, in addition to macrophages, RIPK1 plays a role in the activation of NF-κB in response to inflammatory stimuli in vascular ECs.

Therapeutic Knockdown of RIPK1 Protects Against Early Atherosclerosis

Given our observation that RIPK1 expression is expressed in early lesions in both humans and mice and that RIPK1 promotes NF-κB inflammation in response to atherogenic and inflammatory ligands in vitro, we postulated that RIPK1 inhibition during lesion initiation would reduce atherosclerotic lesion size. To test this, we administered a control or RIPK1 ASO to male and female *Apoe*–/– mice fed a Western diet for 8 weeks (Figure 4). Using antibodies that recognize the 2′-fluoromethoxyethyl modification on the oligonucleotides, we confirmed that ASOs were delivered to the atherosclerotic lesions and demonstrated RIPK1 knockdown in both the intimal lesion and endothelial layer (Figure IIIA in the Data Supplement). Assessment of the en face lesion area in ascending and descending aorta revealed that RIPK1 ASO significantly reduced lesion development in comparison with control ASO-treated mice (58.8% in comparison with control ASO-treated mice (Figure 5A). Furthermore, examination of aortic root lesions demonstrated that RIPK1 ASO-treated mice had a 47% reduction in lesion area in comparison with control ASO (Figure 5B). Using a second ASO directed against RIPK1 with a unique sequence (RIPK1 ASO-B), we also observed significant changes in aortic en face and sinus lesion area (Figure IIIB and IIIC in the Data Supplement). There was no difference in the en face aortic and aortic sinus lesion area between untreated (no treatment) and control ASO-treated mice (Figure IIID in the Data Supplement). There was no significant difference in total plasma cholesterol and body weight (Figure IVA and IVB in the Data Supplement). In addition, the administration of these ASOs in vivo was not toxic as reflected by the lack of differences in circulating liver enzymes, ALT (alanine aminotransferase) and AST (aspartate aminotransferase), between no treatment, control ASO-treated, and RIPK1 ASO-treated mice (Figure IVC in the Data Supplement), and we observed a significant decrease in *Nfkb1* expression in the livers of RIPK1 ASO-treated mice (Figure 5C). To evaluate the systemic inflammatory profile of these mice, we measured the levels of circulating cytokines in the serum, and find a reduction in the proinflammatory cytokines, IL-1α, IL-3, IL-5, IL-5, IL-12(p70), IL-17A, eotaxin, and GM-CSF (Figure 5D), whereas other proinflammatory cytokines, including IL-1β and TNFα, remained unchanged (Figure IVD in the Data Supplement). We assessed lesion composition by immunostaining markers of macrophages (Mac-2) and SMCs (α-smooth muscle actin) within aortic sinus lesions. Consistent with a reduction in atherosclerotic progression, we detected a reduction in macrophage area by 36.92% (RIPK1 ASO, $P<0.01$) and SMC area by 66.5% ($P<0.001$; Figure 5E). Last, we observed a reduction in IL-1α staining in aortic root lesions of mice administered RIPK1-ASO (Figure 5F), which is consistent with the decreased inflammation observed in these mice. Together, these data demonstrate that therapeutic silencing of RIPK1 during atherosclerotic progression induced by a Western diet is dramatically reduced by directly inhibiting NF-κB inflammatory signaling and the secretion of proinflammatory cytokines in the serum.

DISCUSSION

In this study, we find that RIPK1 gene expression is high in early-stage atherosclerotic lesions in mice and humans and is upregulated in proinflammatory macrophages in vitro. In vitro and in vivo, knockdown of RIPK1 in macrophages and ECs reduces inflammatory gene expression in TNFα-stimulated conditions and inhibits NF-κB activity. In macrophages, loss of RIPK1 reduces the secretion of proinflammatory cytokines, IL-1α and IL-1β. In the *Apoe*–/– mouse model of atherosclerosis, which develops large foam cell–rich inflammatory lesions with high-cholesterol diet feeding, systemic RIPK1 knockdown markedly reduces atherosclerotic lesions, with no effect on plasma cholesterol or body weight. Furthermore, both liver inflammatory gene expression and circulating serum inflammatory cytokines are also reduced in RIPK1 ASO-treated mice. Together, our study places RIPK1 at the center of inflammatory signaling during atherosclerosis development (Figure 6).

RIPK1 sits downstream of inflammatory receptors in a complex that coordinates cell survival, cell death, and activation of NF-κB. If the TNFR1 is engaged by TNFα, a membrane-bound complex forms, promoting the modification of RIPK1 by linear K63-ubiquitination, allowing the recruitment of TAK1 (transforming growth factor-β-activated kinase 1) and stabilization of IKKα/IKKβ, degradation of IκBα, and the release and activation of MAPK (mitogen-activated protein kinase) and NF-κB.[18]

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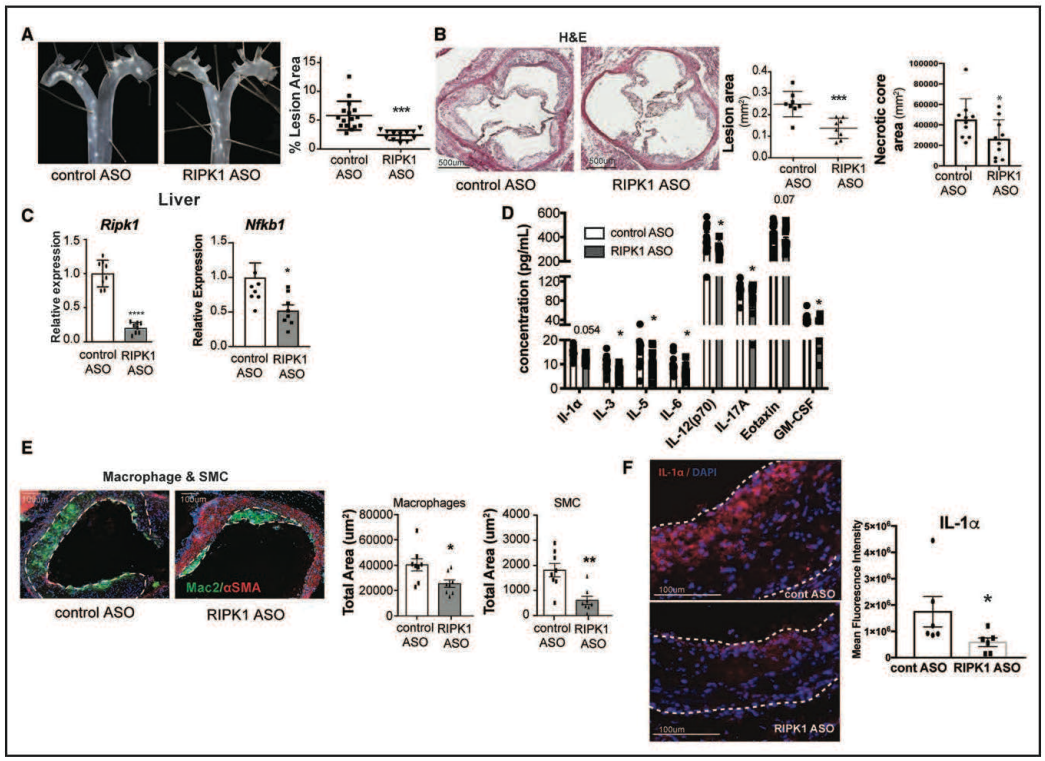


Figure 5. RIPK1 ASO reduces atherosclerotic lesion development in Apoe$^{-/-}$ mice.
Apoe$^{-/-}$ mice were fed a Western diet for 8 weeks and simultaneously treated with control ASO or RIPK1 ASO. **A** and **B**, Quantification of atherosclerotic lesion area. En face lesion area, as a percentage of total area of the aorta, in the aortic arch and descending aorta (**A**) and aortic sinus lesion area across the entire aortic root from H&E-stained sections (**B**); n=9 to 12 mice per group, *P≤0.05, Student t test. **C**, Gene expression of *Ripk1* and *Nfkb1* in the liver of treated mice. **D**, Circulating cytokine levels in the plasma of cont ASO- and RIPK1 ASO-treated mice as determined by the Bioplex Mouse Cytokine Array at the end of the 8-week study; n=9 to 12 mice per treatment group. *P≤0.05, Student t test. **E**, Macrophage (Mac2, green) and SMC (α-smooth muscle actin [αSMA], red) area was quantified in aortic sinus sections from cont ASO- and RIPK1 ASO-treated mice; n=9 to 12 mice per group, *P≤0.05, Student t test. **F**, Immunofluorescent staining for IL-1α in lesions from cont ASO- and RIPK1 ASO-treated mice; n=6 mice per group, *P≤0.05, Mann-Whitney U test. ASO indicates antisense oligonucleotide; cont, control; DAPI, 4′,6-diamidino-2-phenylindole; GM-CSF, granulocyte-macrophage colony-stimulating factor; H&E, hematoxylin and eosin; IL, interleukin; RIPK1, receptor-interacting serine/threonine-protein kinase 1; and SMC, smooth muscle cell.

Prosurvival factors, such as MK2, that are produced by activation of MAPK and NF-κB offset the death receptor signaling through TNFR1 largely because of inactivating the phosphorylation activity of RIPK1, thereby preventing its engagement of the apoptosis and necroptosis pathways.[40–42] The germline deletion of RIPK1 results in early postnatal lethality, which has hampered the ability to study its role in animal models of advanced or chronic disease. Cell-specific deletion of RIPK1 using Cre recombinase and floxed *Ripk1* alleles has revealed that the complete absence of RIPK1 results in cell death of hematopoietic and progenitor cells, and the intestinal epithelium and keratinocytes, as well.[43–46] Humans with mutations in RIPK1 have severe inflammatory disorders attributable to the hyperactivation of RIPK3 and necroptosis.[47–49] Although the full spectrum of RIPK1 functions under different conditions is still unclear, it appears as though RIPK1 acts as a scaffold that, during normal development and at steady state, restricts the inappropriate activation of RIPK3 and inflammatory cell death and enables survival, MAPK and NF-κB signaling. It is surprising that knocking down RIPK1 does not promote spontaneous cell death, nor does it impact the ability of macrophages to undergo necroptosis in response to atherogenic or necroptotic ligands. This implies that, when minimal levels of RIPK1 are maintained, RIPK1 expression favors the activation of the NF-κB pathway in response to atherogenic ligands. This is similar to what is observed using short hairpin RNA to RIPK1 in breast cancer cells, where knockdown of RIPK1 can promote autophagy and cell survival under nutrient-depleted conditions, whereas the complete absence of RIPK1 gene sensitizes cells to cell death.[50,51] Thus, under chronic atherogenic proinflammatory conditions, RIPK1 expression can be reduced to abrogate excessive activation of NF-κB, while maintained at sufficient levels to prevent excessive cell death and further tissue inflammation.

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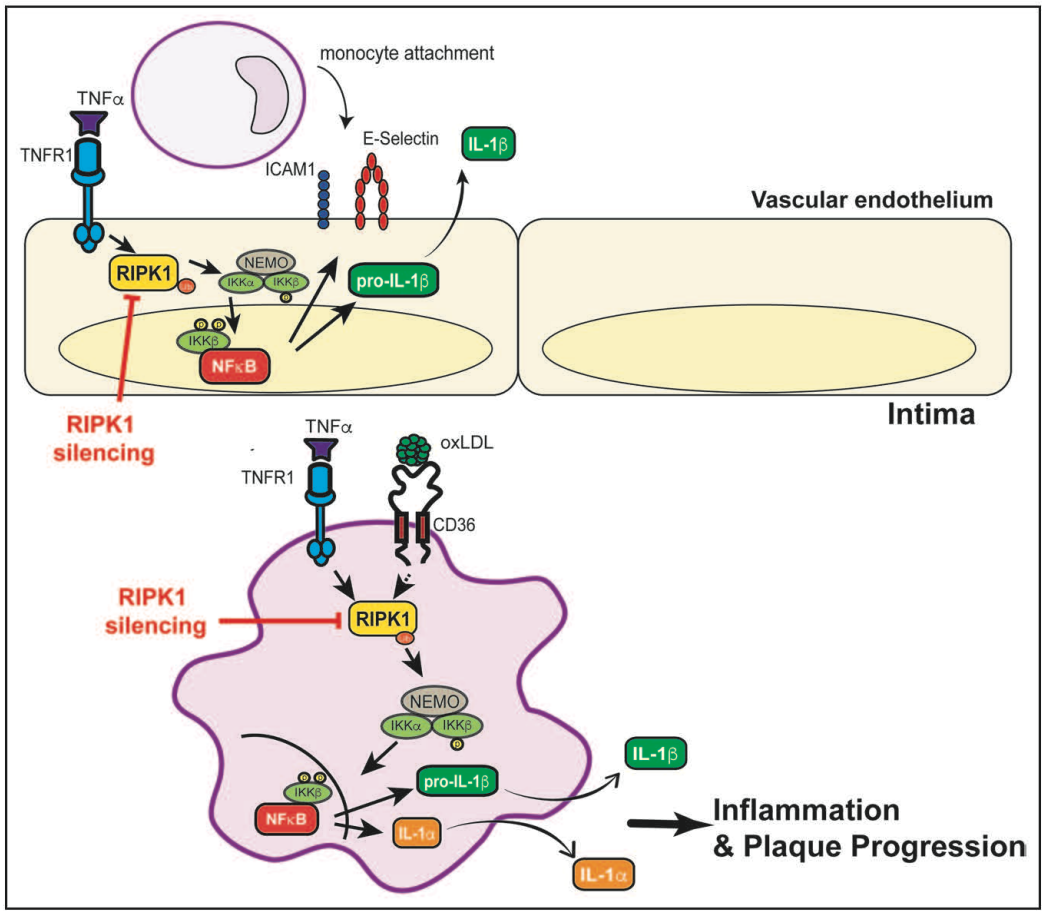


Figure 6. RIPK1 drives NF-κB–dependent activation and cytokine secretion from within atherosclerotic lesions.
In early atherosclerosis, inflammatory stimuli such as TNFα or oxLDL stimulate RIPK1 gene expression. In vascular endothelial cells, activation through TNFα induces adhesion proteins ICAM and E-selectin, which recruits monocytes into the vascular intima. Loss of RIPK1 reduces NF-κB activation, expression of cytokines and adhesion molecules, to reduce monocyte recruitment. In lesional macrophages, loss of RIPK1 reduces NF-κB signaling and IL-1α and IL-1β production and secretion. Together, reduced RIPK1 results in reduced atherosclerotic lesion progression. ICAM-1 indicates intercellular adhesion molecule 1; IL, interleukin; NEMO, NF-kappa-B essential modulator; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; oxLDL, oxidized low-density lipoprotein; RIPK1, receptor-interacting serine/threonine-protein kinase 1; TNFR1, tumor necrosis factor receptor 1; and TNFα, tumor necrosis factor α.

The importance of RIPK3-mediated necroptosis in the atherosclerotic plaque has recently been elucidated, where genetic deletion of *Ripk3* reduced advanced atherosclerotic lesions and necrotic core area in both *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice.[23] In the same way, inhibition of RIPK1-mediated necroptosis by the pharmacological inhibitor Necrostatin-1s reduced lesion complexity and necrotic core area in *Apoe*$^{-/-}$ mice with established atherosclerosis.[10] Together, these data implicate RIPK3-mediated necroptosis in necrotic core formation in advanced, established atherosclerosis. This is in contrast to the role of RIPK2, a related but distinct RIP kinase involved in host defense,[52–54] where *Ripk2* deletion exacerbates atherosclerotic lesion development.[52] In contrast to the other members of the RIP kinase family, the role for RIPK1 in initiating atherosclerosis was unknown. In this study, we find robust RIPK1 gene expression within early atherosclerotic lesions in both mice and humans, where it correlates strongly with other metrics of inflammation, namely IKK and TNFα. In other words, lesional RIPK1 expression is high when inflammation is most active in promoting lesion formation. This is distinct from what is observed for phosphorylated MLKL, an effector of necroptotic cell death, that we found was uniquely expressed in large, advanced atherosclerotic plaques in humans and virtually absent in smaller, less complex lesions.[10] Others have shown that TNFα expression and activity is also highest in early atherosclerotic lesions.[55] For these reasons, we

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delivered antisense inhibitors targeting RIPK1 to mice during the initiation phases of lesion formation, when we predicted RIPK1 would be at its highest level. Similar to TNFα and RIPK1 expression, NF-κB expression has been linked to early events in the initiation of atherosclerosis, where it is expressed in SMCs and macrophages and is elevated in ECs in atherosclerosis-prone regions.[56–58] Unlike in ECs,[59] the complete loss of NF-κB activity in macrophages does not reduce atherosclerosis and, in fact, promotes necrotic core expansion attributable to massive cell death of macrophages.[60] This implies that, not unlike RIPK1, basal levels of NF-κB are required to restrict uncontrolled cell death. In contrast, studies that use specific NF-κB inhibitors find that turning down NF-κB, but not completely inactivating it, reduces inflammatory lesions and protects against atherosclerosis.[61,62] Our data agree with the notion that activation of the RIPK1-NF-κB axis early in lesion formation is a key driver of atherogenesis, in particular, in ECs, but that the basal activity of this pathway is necessary to avoid uncontrolled cell death and necrosis within lesions. Although we did not investigate the cell-specific roles for RIPK1 in driving atherogenesis, we speculate that RIPK1 knockdown could beneficially impact atherosclerosis development through both lesional macrophages and vascular ECs, because both of these cell types are targeted by the ASOs used in this study. However, because complete loss of RIPK1 in barrier tissues leads to unrestricted cell death and exacerbates inflammation, the contribution of endothelial versus macrophage RIPK1 to atherosclerosis remains to be investigated.[45,46]

The concept of therapeutic targeting of inflammation to reduce cardiovascular disease risk has now been validated with the recent results of the CANTOS trial, where an IL-1β blocking antibody reduced adverse cardiovascular events by 15% in patients with low circulating LDL cholesterol.[15] Likewise, the COLCOT trial showed a significant reduction with the anti-inflammatory compound colchicine in patients that had a previous myocardial infarction.[16] This indicates that, even with appropriate cholesterol management, inflammatory pathways persist and can be blocked to further prevent disease progression. However, blocking IL-1β led to a significant increase in lethal infections, likely because of the important role IL-1β plays in the host response to pathogens. We found reduced macrophage secretion of IL-1α and IL-1β with RIPK1 ASO treatment. Both IL-1β and IL-1α, which are downstream of NF-κB activation, directly contribute to atherosclerotic lesion formation in mice,[5] and we find that levels of IL-1α are decreased on RIPK1 inhibition in both the serum and the lesion. Although the maturation of pro-IL-1β is dependent on NLRP3 inflammasome activation and caspase-1 activity,[4] IL-1α activation can be independent of these processes.[5]We have consistently shown that the inhibition or deletion of RIPK3, downstream of RIPK1, in advanced atherosclerotic plaques does not affect circulating IL-1β yet reduces advanced lesion development.[10,37] Moreover, recent studies have revealed a role for IL-1β in the stabilization of atherosclerotic plaques by activation of SMC responses, further calling into question the need to develop alternative anti-inflammatory therapies without complete abrogation of IL-1β activity.[63] Because RIPK1 expression is elevated directly in the plaque in both mice and humans, and antisense approaches like those used in our study can target lesion burden without systemic changes in IL-1β, we argue that targeting RIPK1 may be better suited to reduce inflammation in the atherosclerotic plaque while leaving host innate immune defenses intact.

In summary, we find that RIPK1 expression is high in plaques that can be classified as early and less advanced, likely when inflammatory indices are also elevated. In vitro, proinflammatory M1 macrophages express higher levels of RIPK1 than both M0 and M2, and, as such, reducing RIPK1 gene expression leads to a reduction in NF-κB gene activation and secretion of IL-1α in vivo, which is a key driver of atherosclerosis. In *Apoe*$^{-/-}$ mice fed a high cholesterol diet for 8 weeks, RIPK1 ASOs reduced lesion progression and macrophage accumulation in comparison with control mice. Together these data implicate RIPK1 as a hub of inflammatory gene activation directly in the atherosclerotic plaque and a potentially important future therapeutic target for residual inflammatory risk in patients with high risk of developing coronary and other vascular diseases.

ARTICLE INFORMATION

Received October 25, 2018; accepted October 23, 2020.

The Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/CIRCULATIONAHA.118.038379.

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Acknowledgments

The authors acknowledge the Animal Care and Veterinary Services at the University of Ottawa Heart Institute for their expertise; X. Zhao for her technical assistance, and University of Queensland Biological Resources. Dr Karunakaran, H. Wyatt, J.W. Kandiah, and Dr Mompeon performed in vitro assays. Dr Karunakaran, Dr Nguyen, M. Geoffrion, and Z. Lister performed the animal studies and analysis. Drs Jung, Alenghat, Guo, Kolodgie, Virmani, and Rayner performed human sample analysis. Dr Lee provided the antisense oligonucleotides. Drs Matic and Hedin contributed data from the BiKE Project (Biobank of Karolinska Endarterectomies). Drs Cheng and Fish performed gene expression analysis in *Ldlr–/–* mice aortas. Drs Pan and Lusis performed analyses from the Hybrid Mouse Diversity Panel data set. Drs Vreeken and van Gils performed the endothelial cell studies. Drs Karunakaran and Rayner wrote the article with input from all authors.

Sources of Funding

This study was supported with funding from the following organizations: Canadian Institutes of Health Research (to Dr Rayner), National Institutes of Health (R01 HL119047 to Dr Rayner, HL147883 and HL144651 to Dr Lusis), European Research Area Network on Cardiovascular Diseases (to Dr Rayner and van Gils), National Psoriasis Foundation (to Dr Karunakaran) and the University of Queensland (to Dr Karunakaran) The BiKE Study was conducted with support from the Swedish Heart and Lung Foundation, the Swedish Research Council (K2009-65X-2233-01-3, K2013-65X-06816-30-4, and 349-2007-8703), Uppdrag Besegra Stroke (P581/2011-123), the Strategic Cardiovascular Programs of Karolinska Institutet and Stockholm County Council, the Stockholm County Council (ALF2011-0260 and ALF-2011-0279) and the Foundation for Strategic Research. Dr Matic is the recipient of fellowships from the Swedish Society for Medical Research and the Swedish Heart and Lung Foundation. Dr Gordon was supported by a National Health and Medical Research Council Project Grant (APP1158002) and an Australian Research Council Discovery Project (DP200100737). Dr Lusis was supported by le Foundation Leduc. Dr van Gils was supported by the Dutch Heart Foundation (2013T1127). Drs van Gils and Rayner were supported by the MiSS-CVD (European Research Area Network on Cardiovascular Diseases).

Disclosures

Dr Lee is an employee of Ionis Pharmaceuticals. The other authors have no other disclosures.

Supplemental Materials

Data Supplement Figures I–IV

REFERENCES

1. Tabas I, Lichtman AH. Monocyte-macrophages and T cells in atherosclerosis. *Immunity*. 2017;47:621–634. doi: 10.1016/j.immuni.2017.09.008
2. Tabas I, Bornfeldt KE. Macrophage phenotype and function in different stages of atherosclerosis. *Circ Res*. 2016;118:653–667. doi: 10.1161/CIRCRESAHA.115.306256
3. Collins T, Cybulsky MI. NF-kappaB: pivotal mediator or innocent bystander in atherogenesis? *J Clin Invest*. 2001;107:255–264. doi: 10.1172/JCI10373
4. Sheedy FJ, Grebe A, Rayner KJ, Kalantari P, Ramkhelawon B, Carpenter SB, Becker CE, Ediriweera HN, Mullick AE, Golenbock DT, et al. CD36 coordinates NLRP3 inflammasome activation by facilitating intracellular nucleation of soluble ligands into particulate ligands in sterile inflammation. *Nat Immunol*. 2013;14:812–820. doi: 10.1038/ni.2639
5. Freigang S, Ampenberger F, Weiss A, Kanneganti TD, Iwakura Y, Hersberger M, Kopf M. Fatty acid-induced mitochondrial uncoupling elicits inflammasome-independent IL-1α and sterile vascular inflammation in atherosclerosis. *Nat Immunol*. 2013;14:1045–1053. doi: 10.1038/ni.2704
6. Crisby M, Kallin B, Thyberg J, Zhivotovsky B, Orrenius S, Kostulas V, Nilsson J. Cell death in human atherosclerotic plaques involves both oncosis and apoptosis. *Atherosclerosis*. 1997;130:17–27. doi: 10.1016/s0021-9150(96)06037-6
7. Liu J, Thewke DP, Su YR, Linton MF, Fazio S, Sinensky MS. Reduced macrophage apoptosis is associated with accelerated atherosclerosis in low-density lipoprotein receptor-null mice. *Arterioscler Thromb Vasc Biol*. 2005;25:174–179. doi: 10.1161/01.ATV.0000148548.47755.22
8. Arai S, Shelton JM, Chen M, Bradley MN, Castrillo A, Bookout AL, Mak PA, Edwards PA, Mangelsdorf DJ, Tontonoz P, et al. A role for the apoptosis inhibitory factor AIM/Spalpha/Api6 in atherosclerosis development. *Cell Metab*. 2005;1:201–213. doi: 10.1016/j.cmet.2005.02.002
9. Rayner KJ. Cell death in the vessel wall: the good, the bad, the ugly. *Arterioscler Thromb Vasc Biol*. 2017;37:e75–e81. doi: 10.1161/ATVBAHA.117.309229
10. Karunakaran D, Geoffrion M, Wei L, Gan W, Richards L, Shangari P, DeKemp EM, Beanlands RA, Perisic L, Maegdefessel L, et al. Targeting macrophage necroptosis for therapeutic and diagnostic interventions in atherosclerosis. *Sci Adv*. 2016;2:e1600224. doi: 10.1126/sciadv.1600224
11. Kasikara C, Doran AC, Cai B, Tabas I. The role of non-resolving inflammation in atherosclerosis. *J Clin Invest*. 2018;128:2713–2723. doi: 10.1172/JCI97950
12. Yurdagul A Jr, Doran AC, Cai B, Fredman G, Tabas IA. Mechanisms and consequences of defective efferocytosis in atherosclerosis. *Front Cardiovasc Med*. 2017;4:86. doi: 10.3389/fcvm.2017.00086
13. Baigent C, Blackwell L, Emberson J, Holland LE, Reith C, Bhala N, Peto R, Barnes EH, Keech A, Simes J, et al; Cholesterol Treatment Trialists' (CTT) Collaboration. Efficacy and safety of more intensive lowering of LDL cholesterol: a meta-analysis of data from 170,000 participants in 26 randomised trials. *Lancet*. 2010;376:1670–1681. doi: 10.1016/S0140-6736(10)61350-5
14. Kingwell BA, Chapman MJ, Kontush A, Miller NE. HDL-targeted therapies: progress, failures and future. *Nat Rev Drug Discov*. 2014;13:445–464. doi: 10.1038/nrd4279
15. Ridker PM, Everett BM, Thuren T, MacFadyen JG, Chang WH, Ballantyne C, Fonseca F, Nicolau J, Koenig W, Anker SD, et al; CANTOS Trial Group. Antiinflammatory therapy with canakinumab for atherosclerotic disease. *N Engl J Med*. 2017;377:1119–1131. doi: 10.1056/NEJMoa1707914
16. Tardif JC, Kouz S, Waters DD, Bertrand OF, Diaz R, Maggioni AP, Pinto FJ, Ibrahim R, Gamra H, Kiwan GS, et al. Efficacy and safety of low-dose colchicine after myocardial infarction. *N Engl J Med*. 2019;381:2497–2505. doi: 10.1056/NEJMoa1912388
17. Silke J, Rickard JA, Gerlic M. The diverse role of RIP kinases in necroptosis and inflammation. *Nat Immunol*. 2015;16:689–697. doi: 10.1038/ni.3206
18. Dondelinger Y, Darding M, Bertrand MJ, Walczak H. Poly-ubiquitination in TNFR1-mediated necroptosis. *Cell Mol Life Sci*. 2016;73:2165–2176. doi: 10.1007/s00018-016-2191-4
19. Moquin DM, McQuade T, Chan FK. CYLD deubiquitinates RIP1 in the TNFα-induced necrosome to facilitate kinase activation and programmed necrosis. *PLoS One*. 2013;8:e76841. doi: 10.1371/journal.pone.0076841
20. Cuny GD, Degterev A. RIPK protein kinase family: atypical lives of typical kinases [published online July 27, 2020]. *Semin Cell Dev Biol*. doi: 10.1016/j.semcdb.2020.06.014. https://www.sciencedirect.com/science/article/abs/pii/S1084952119302447
21. Petrie EJ, Czabotar PE, Murphy JM. The structural basis of necroptotic cell death signaling. *Trends Biochem Sci*. 2019;44:53–63. doi: 10.1016/j.tibs.2018.11.002
22. Karunakaran D, Geoffrion M, Wei L, Gan W, Richards L, Shangari P, DeKemp EM, Beanlands RA, Perisic L, Maegdefessel L, et al. Targeting macrophage necroptosis for therapeutic and diagnostic interventions in atherosclerosis. *Sci Adv*. 2016;2:e1600224. doi: 10.1126/sciadv.1600224
23. Lin J, Li H, Yang M, Ren J, Huang Z, Han F, Huang J, Ma J, Zhang D, Zhang Z, et al. A role of RIP3-mediated macrophage necrosis in atherosclerosis development. *Cell Rep*. 2013;3:200–210. doi: 10.1016/j.celrep.2012.12.012
24. Nguyen MA, Karunakaran D, Geoffrion M, Cheng HS, Tandoc K, Perisic Matic L, Hedin U, Maegdefessel L, Fish JE, Rayner KJ. Extracellular vesicles secreted by atherogenic macrophages transfer microRNA to inhibit cell migration. *Arterioscler Thromb Vasc Biol*. 2018;38:49–63. doi: 10.1161/ATVBAHA.117.309795
25. Naylor AR, Rothwell PM, Bell PR. Overview of the principal results and secondary analyses from the European and North American randomised trials of endarterectomy for symptomatic carotid stenosis. *Eur J Vasc Endovasc Surg*. 2003;26:115–129. doi: 10.1053/ejvs.2002.1946
26. Perisic L, Aldi S, Sun Y, Folkersen L, Razuvaev A, Roy J, Lengquist M, Åkesson S, Wheelock CE, Maegdefessel L, et al. Gene expression signatures, pathways and networks in carotid atherosclerosis. *J Intern Med*. 2016;279:293–308. doi: 10.1111/joim.12448

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27. Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res.* 2002;30:207–210. doi: 10.1093/nar/30.1.207
28. Ayari H, Bricca G. Identification of two genes potentially associated in iron-heme homeostasis in human carotid plaque using microarray analysis. *J Biosci.* 2013;38:311–315. doi: 10.1007/s12038-013-9310-2
29. Arking DE, Junttila MJ, Goyette P, Huertas-Vazquez A, Eijgelsheim M, Blom MT, Newton-Cheh C, Reinier K, Teodorescu C, Uy-Evanado A, et al. Identification of a sudden cardiac death susceptibility locus at 2q24.2 through genome-wide association in European ancestry individuals. *PLoS Genet.* 2011;7:e1002158. doi: 10.1371/journal.pgen.1002158
30. Berger SB, Kasparcova V, Hoffman S, Swift B, Dare L, Schaeffer M, Capriotti C, Cook M, Finger J, Hughes-Earle A, et al. Cutting edge: RIP1 kinase activity is dispensable for normal development but is a key regulator of inflammation in SHARPIN-deficient mice. *J Immunol.* 2014;192:5476–5480. doi: 10.4049/jimmunol.1400499
31. Sun X, He S, Wara AKM, Icli B, Shvartz E, Tesmenitsky Y, Belkin N, Li D, Blackwell TS, Sukhova GK, et al. Systemic delivery of microRNA-181b inhibits nuclear factor-κB activation, vascular inflammation, and atherosclerosis in apolipoprotein E-deficient mice. *Circ Res.* 2014;114:32–40. doi: 10.1161/CIRCRESAHA.113.302089
32. Lusis AJ, Seldin MM, Allayee H, Bennett BJ, Civelek M, Davis RC, Eskin E, Farber CR, Hui S, Mehrabian M, et al. The Hybrid Mouse Diversity Panel: a resource for systems genetics analyses of metabolic and cardiovascular traits. *J Lipid Res.* 2016;57:925–942. doi: 10.1194/jlr.R066944
33. Bennett BJ, Davis RC, Civelek M, Orozco L, Wu J, Qi H, Pan C, Packard RR, Eskin E, Yan M, et al. Genetic architecture of atherosclerosis in mice: a systems genetics analysis of common inbred strains. *PLoS Genet.* 2015;11:e1005711. doi: 10.1371/journal.pgen.1005711
34. Cheng HS, Besla R, Li A, Chen Z, Shikatani EA, Nazari-Jahantigh M, Hammoutène A, Nguyen MA, Geoffrion M, Cai L, et al. Paradoxical suppression of atherosclerosis in the absence of microRNA-146a. *Circ Res.* 2017;121:354–367. doi: 10.1161/CIRCRESAHA.116.310529
35. Karunakaran D, Richards L, Geoffrion M, Barrette D, Gotfrit RJ, Harper ME, Rayner KJ. Therapeutic inhibition of miR-33 promotes fatty acid oxidation but does not ameliorate metabolic dysfunction in diet-induced obesity. *Arterioscler Thromb Vasc Biol.* 2015;35:2536–2543. doi: 10.1161/ATVBAHA.115.306404
36. Rayner KJ, Sheedy FJ, Esau CC, Hussain FN, Temel RE, Parathath S, van Gils JM, Rayner AJ, Chang AN, Suarez Y, et al. Antagonism of miR-33 in mice promotes reverse cholesterol transport and regression of atherosclerosis. *J Clin Invest.* 2011;121:2921–2931. doi: 10.1172/JCI57275
37. Meng L, Jin W, Wang X. RIP3-mediated necrotic cell death accelerates systematic inflammation and mortality. *Proc Natl Acad Sci USA.* 2015;112:11007–11012. doi: 10.1073/pnas.1514730112
38. Newton K. RIPK1 and RIPK3: critical regulators of inflammation and cell death. *Trends Cell Biol.* 2015;25:347–353. doi: 10.1016/j.tcb.2015.01.001
39. Sun X, Sit A, Feinberg MW. Role of miR-181 family in regulating vascular inflammation and immunity. *Trends Cardiovasc Med.* 2014;24:105–112. doi: 10.1016/j.tcm.2013.09.002
40. Dondelinger Y, Delanghe T, Rojas-Rivera D, Priem D, Delvaeye T, Bruggeman I, Van Herreweghe F, Vandenabeele P, Bertrand MJM. MK2 phosphorylation of RIPK1 regulates TNF-mediated cell death. *Nat Cell Biol.* 2017;19:1237–1247. doi: 10.1038/ncb3608
41. Jaco I, Annibaldi A, Lalaoui N, Wilson R, Tenev T, Laurien L, Kim C, Jamal K, Wicky John S, Liccardi G, et al. MK2 phosphorylates RIPK1 to prevent TNF-induced cell death. *Mol Cell.* 2017;66:698–710.e5. doi: 10.1016/j.molcel.2017.05.003
42. Menon MB, Gropengießer J, Fischer J, Novikova L, Deuretzbacher A, Lafera J, Schimmeck H, Czymmeck N, Ronkina N, Kotlyarov A, et al. p38MAPK/MK2-dependent phosphorylation controls cytotoxic RIPK1 signalling in inflammation and infection. *Nat Cell Biol.* 2017;19:1248–1259. doi: 10.1038/ncb3614
43. Kelliher MA, Grimm S, Ishida Y, Kuo F, Stanger BZ, Leder P. The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. *Immunity.* 1998;8:297–303. doi: 10.1016/s1074-7613(00)80535-x
44. Zhang H, Zhou X, McQuade T, Li J, Chan FK, Zhang J. Functional complementation between FADD and RIP1 in embryos and lymphocytes. *Nature.* 2011;471:373–376. doi: 10.1038/nature09878
45. Dannappel M, Vlantis K, Kumari S, Polykratis A, Kim C, Wachsmuth L, Eftychi C, Lin J, Corona T, Hermance N, et al. RIPK1 maintains epithelial homeostasis by inhibiting apoptosis and necroptosis. *Nature.* 2014;513:90–94. doi: 10.1038/nature13608
46. Takahashi N, Vereecke L, Bertrand MJ, Duprez L, Berger SB, Divert T, Gonçalves A, Sze M, Gilbert B, Kourula S, et al. RIPK1 ensures intestinal homeostasis by protecting the epithelium against apoptosis. *Nature.* 2014;513:95–99. doi: 10.1038/nature13706
47. Lalaoui N, Boyden SE, Oda H, Wood GM, Stone DL, Chau D, Liu L, Stoffels M, Kratina T, Lawlor KE, et al. Mutations that prevent caspase cleavage of RIPK1 cause autoinflammatory disease. *Nature.* 2020;577:103–108. doi: 10.1038/s41586-019-1828-5
48. Li Y, Führer M, Bahrami E, Socha P, Klaudel-Dreszler M, Bouzidi A, Liu Y, Lehle AS, Magg T, Hollizeck S, et al. Human RIPK1 deficiency causes combined immunodeficiency and inflammatory bowel diseases. *Proc Natl Acad Sci USA.* 2019;116:970–975. doi: 10.1073/pnas.1813582116
49. Cuchet-Lourenço D, Eletto D, Wu C, Plagnol V, Papapietro O, Curtis J, Ceron-Gutierrez L, Bacon CM, Hackett S, Alsaleem B, et al. Biallelic RIPK1 mutations in humans cause severe immunodeficiency, arthritis, and intestinal inflammation. *Science.* 2018;361:810–813. doi: 10.1126/science.aar2641
50. Yonekawa T, Gamez G, Kim J, Tan AC, Thorburn J, Gump J, Thorburn A, Morgan MJ. RIP1 negatively regulates basal autophagic flux through TFEB to control sensitivity to apoptosis. *EMBO Rep.* 2015;16:700–708. doi: 10.15252/embr.201439496
51. Green DR. Another face of RIPK1. *EMBO Rep.* 2015;16:674–675. doi: 10.15252/embr.201540470
52. Levin MC, Jirholt P, Wramstedt A, Johansson ME, Lundberg AM, Trajkovska MG, Ståhlman M, Fogelstrand P, Brisslert M, Fogelstrand L, et al. Rip2 deficiency leads to increased atherosclerosis despite decreased inflammation. *Circ Res.* 2011;109:1210–1218. doi: 10.1161/CIRCRESAHA.111.246702
53. Shimada K, Chen S, Dempsey PW, Sorrentino R, Alsabeh R, Slepenkin AV, Peterson E, Doherty TM, Underhill D, Crother TR, et al. The NOD/RIP2 pathway is essential for host defenses against *Chlamydophila pneumoniae* lung infection. *PLoS Pathog.* 2009;5:e1000379. doi: 10.1371/journal.ppat.1000379
54. Sukumaran B, Ogura Y, Pedra JH, Kobayashi KS, Flavell RA, Fikrig E. Receptor interacting protein-2 contributes to host defense against *Anaplasma phagocytophilum* infection. *FEMS Immunol Med Microbiol.* 2012;66:211–219. doi: 10.1111/j.1574-695X.2012.01001.x
55. Xiao N, Yin M, Zhang L, Qu X, Du H, Sun X, Mao L, Ren G, Zhang C, Geng Y, et al. Tumor necrosis factor-alpha deficiency retards early fatty-streak lesion by influencing the expression of inflammatory factors in apoE-null mice. *Mol Genet Metab.* 2009;96:239–244. doi: 10.1016/j.ymgme.2008.11.166
56. de Winther MP, Kanters E, Kraal G, Hofker MH. Nuclear factor kappaB signaling in atherogenesis. *Arterioscler Thromb Vasc Biol.* 2005;25:904–914. doi: 10.1161/01.ATV.0000160340.72641.87
57. Brand K, Page S, Rogler G, Bartsch A, Brandl R, Knuechel R, Page M, Kaltschmidt C, Baeuerle PA, Neumeier D. Activated transcription factor nuclear factor-kappa B is present in the atherosclerotic lesion. *J Clin Invest.* 1996;97:1715–1722. doi: 10.1172/JCI118598
58. Hajra L, Evans AI, Chen M, Hyduk SJ, Collins T, Cybulsky MI. The NF-kappa B signal transduction pathway in aortic endothelial cells is primed for activation in regions predisposed to atherosclerotic lesion formation. *Proc Natl Acad Sci USA.* 2000;97:9052–9057. doi: 10.1073/pnas.97.16.9052
59. Gareus R, Kotsaki E, Xanthoulea S, van der Made I, Gijbels MJ, Kardakaris R, Polykratis A, Kollias G, de Winther MP, Pasparakis M. Endothelial cell-specific NF-kappaB inhibition protects mice from atherosclerosis. *Cell Metab.* 2008;8:372–383. doi: 10.1016/j.cmet.2008.08.016
60. Kanters E, Pasparakis M, Gijbels MJ, Vergouwe MN, Partouns-Hendriks I, Fijneman RJ, Clausen BE, Förster I, Kockx MM, Rajewsky K, et al. Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice. *J Clin Invest.* 2003;112:1176–1185. doi: 10.1172/JCI18580
61. Zhang Y, Yang X, Bian F, Wu P, Xing S, Xu G, Li W, Chi J, Ouyang C, Zheng T, et al. TNF-α promotes early atherosclerosis by increasing transcytosis of LDL across endothelial cells: crosstalk between NF-κB and PPAR-γ. *J Mol Cell Cardiol.* 2014;72:85–94. doi: 10.1016/j.yjmcc.2014.02.012
62. Mallavia B, Recio C, Oguiza A, Ortiz-Muñoz G, Lazaro I, Lopez-Parra V, Lopez-Franco O, Schindler S, Depping R, Egido J, et al. Peptide inhibitor of NF-κB translocation ameliorates experimental atherosclerosis. *Am J Pathol.* 2013;182:1910–1921. doi: 10.1016/j.ajpath.2013.01.022
63. Gomez D, Baylis RA, Durgin BG, Newman AAC, Alencar GF, Mahan S, St Hilaire C, Müller W, Waisman A, Francis SE, et al. Interleukin-1β has atheroprotective effects in advanced atherosclerotic lesions of mice. *Nat Med.* 2018;24:1418–1429. doi: 10.1038/s41591-018-0124-5

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Appendix C

Transradial versus transfemoral access for percutaneous coronary intervention in ST-elevation myocardial infarction: a systematic review and meta-analysis

Citation information:

Di Santo P, Simard T, Wells GA, **Jung RG**, Ramirez FD, Boland P, Marbach JA, Parlow S, Kyeremanteng K, Coyle D, Fergusson D, Russo JJ, Chong AY, So DY, Dick A, Glover C, Labinaz M, Hibbert B, Le May M. Transradial versus transfemoral access for percutaneous coronary intervention in ST-elevation myocardial infarction: a systematic review and meta-analysis. (**2021**). *Circulation: Cardiovascular Interventions*.

Circulation: Cardiovascular Interventions

ORIGINAL ARTICLE

Transradial Versus Transfemoral Access for Percutaneous Coronary Intervention in ST-Segment–Elevation Myocardial Infarction

A Systematic Review and Meta-Analysis

Pietro Di Santo, MD*; Trevor Simard, MD*; George A. Wells, PhD; Richard G. Jung, BSc; F. Daniel Ramirez, MD, MSc; Paul Boland, MD, PhD; Jeffrey A. Marbach, MBBS, MSc; Simon Parlow, MD; Kwadwo Kyeremanteng, MD, MHA; Doug Coyle, MA, MSc, PhD; Dean Fergusson, PhD; Juan J. Russo, MD; Aun-Yeong Chong, MD; Michael Froeschl, MDCM, MSc; Derek Y. So, MD, MSc; Alexander Dick, MD; Christopher Glover, MD; Marino Labinaz, MD; Benjamin Hibbert, MD, PhD; Michel Le May, MD

BACKGROUND: Transradial access (TRA) has emerged as the preferred vascular access site for coronary angiography and percutaneous coronary intervention. This systematic review and meta-analysis was performed to evaluate 30-day all-cause mortality comparing TRA with transfemoral access for percutaneous coronary intervention in patients with ST-segment–elevation myocardial infarction.

METHODS: We performed a systematic literature search and meta-analysis of randomized controlled studies published from inception until January 7, 2020, in MEDLINE, EMBASE, Cochrane Central Register of Controlled Trials, and Web of Science Core Collection. Preferred Reported Items for Systematic Reviews and Meta-Analyses guidelines were used for abstracting data. The primary outcome was all-cause mortality at 30 days. Secondary outcomes included myocardial infarction, major bleeding, stroke, and access site complications.

RESULTS: A total of 14 studies representing 11 707 patients (5802 patients with TRA; 5905 patients with transfemoral access) were included in this systematic review. All-cause mortality (N=8 studies) was significantly reduced in the TRA group with an overall risk ratio (RR) of 0.72 (95% CI, 0.56–0.92) in the pooled analysis. Major bleeding (N=12 studies; RR, 0.60 [95% CI, 0.45–0.80]) and access site complications (N=9 studies; RR, 0.40 [95% CI, 0.30–0.53]) were significantly higher in the transfemoral access group. There was no statistical difference in reinfarction (N=10 studies; RR, 0.96 [95% CI, 0.75–1.25]) or stroke (N=8 studies; RR, 1.47 [95% CI, 0.87–2.50]).

CONCLUSIONS: TRA is associated with lower 30-day mortality, major bleeding, and access site complications when compared with transfemoral access in ST-segment–elevation myocardial infarction patients who undergo percutaneous coronary intervention.

REGISTRATION: URL: https://www.crd.york.ac.uk/PROSPERO/; Unique identifier: 127955.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: acute coronary syndrome ■ coronary angiography ■ glycoprotein ■ percutaneous coronary intervention ■ ST-segment–elevation myocardial infarction

See Editorial by Jolly and Nolan

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*P. Di Santo and T. Simard contributed equally.

The Data Supplement is available at https://www.ahajournals.org/doi/suppl/10.1161/CIRCINTERVENTIONS.120.009994.

For Sources of Funding and Disclosures, see page 268.

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Appendix D

Transcatheter mitral-valve repair in cardiogenic shock and severe mitral regurgitation: a patient-level multi-center analysis

Citation information:

Jung RG, Simard T, Kovach C, Flint K, Don C, Di Santo P, Adamo M, Branca L, Valentini F, Benito-Gonzalez T, Vazquez FF, Estevez-Loureiro R, Berardini A, Conti N, Rapezzi C, Biagini E, Parlow S, Shorr R, Levi A, Manovel A, Cardenal-Piris R, Diaz JF, Shuvy M, Haberman D, Alessandra S, Marini C, Bargagna M, Schiavi D, Paolo D, Buzzatti N, Markovic S, Chan V, Hynes M, Mesana T, Labinaz M, Markovic S, Pappalardo F, Taramasso M, Hibbert B. Transcatheter mitral-valve repair in cardiogenic shock and severe mitral regurgitation: a patient-level multi-center analysis. (**2021**). *JACC: Cardiovascular Interventions*.

JACC: CARDIOVASCULAR INTERVENTIONS VOL. 14, NO. 1, 2021
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NEW RESEARCH PAPERS

STRUCTURAL

Transcatheter Mitral Valve Repair in Cardiogenic Shock and Mitral Regurgitation

A Patient-Level, Multicenter Analysis

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ABSTRACT

OBJECTIVES The aim of this study was to evaluate the outcome of transcatheter mitral valve repair (TMVr) in patients with cardiogenic shock and significant mitral regurgitation (MR).

BACKGROUND Patients in cardiogenic shock with severe MR have a poor prognosis in the setting of conventional medical therapy. Because of its favorable safety profile, TMVr is being increasingly used as an acute therapy in this population, though its efficacy remains unknown.

METHODS A multicenter, collaborative, patient-level analysis was conducted. Patients with cardiogenic shock and moderate to severe (3+) or severe (4+) MR who were not surgical candidates were treated with TMVr. The primary outcome was in-hospital mortality. Secondary outcomes included 90-day mortality, heart failure (HF) hospitalization, and the combined event rate of 90-day mortality and HF hospitalization following dichotomization by TMVr device success.

RESULTS Between January 2011 and February 2019, 141 patients across 14 institutions met the inclusion criteria. In-hospital mortality occurred in 22 patients (15.6%), at 90 days in 38 patients (29.5%), and at one year in 55 patients (42.6%). Median length of hospital stay following TMVr was 10 days (interquartile range: 6 to 20 days). HF hospitalization occurred in 26 patients (18.4%) at a median of 73 days (interquartile range: 26 to 546 days). When stratified by TMVr procedural results, successful TMVr reduced rates of in-hospital mortality (hazard ratio [HR]: 0.36; 95% confidence interval [CI]: 0.13 to 0.98; p = 0.04), 90-day mortality (HR: 0.36; 95% CI: 0.16 to 0.78; p = 0.01), and the composite of 90-day mortality and HF hospitalization (HR: 0.41; 95% CI: 0.19 to 0.90; p = 0.03).

CONCLUSIONS TMVr may improve short- and intermediate-term mortality in high-risk patients with cardiogenic shock and moderate to severe MR. Randomized studies are needed to definitively establish MR as a therapeutic target in patients with cardiogenic shock. (J Am Coll Cardiol Intv 2021;14:1–11) © 2021 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

ISSN 1936-8798 https://doi.org/10.1016/j.jcin.2020.08.037

ABBREVIATIONS AND ACRONYMS

CI = confidence interval

HF = heart failure

HR = hazard ratio

IQR = interquartile range

LVEF = left ventricular ejection fraction

MCS = mechanical circulatory support

MR = mitral regurgitation

SCAI = Society for Cardiovascular Angiography and Interventions

TMVr = transcatheter mitral valve repair

Transcatheter mitral valve repair (TMVr) is an established therapy for patients with functional mitral regurgitation (MR) in the setting of heart failure (HF) or degenerative MR in patients at high or prohibitive surgical risk (1,2). Landmark trials, including EVEREST II (Endovascular Valve Edge-to-Edge Repair Study), COAPT (Cardiovascular Outcomes Assessment of the MitraClip Percutaneous Therapy for Heart Failure Patients With Functional Mitral Regurgitation), and MITRA-FR (Percutaneous Repair with the MitraClip Device for Severe Functional/Secondary Mitral Regurgitation), examined TMVr in patients in stable condition with chronic MR on guideline-directed medical therapy, demonstrating an excellent procedural safety profile (1–4).

Moderate or greater MR is present in 5% to 10% of patients in cardiogenic shock (5,6). The role for TMVr in this setting remains unclear, particularly with the dynamic nature of functional MR in this setting. Importantly, these patients are often at prohibitive risk for surgical intervention and are not candidates for advanced mechanical circulatory support (MCS), representing a population presently without established therapies (7). The role of TMVr as an adjunct therapy in patients with cardiogenic shock remains poorly described (8). Recently, a number of small single-center studies have used TMVr in patients with concurrent MR and shock, supporting it as a safe and feasible alternative (7,9,10).

Herein, we performed a systematic review of the use of TMVr in patients with cardiogenic shock and moderate to severe (3+) or severe (4+) MR to identify all reports. Given the relatively small cohort of patients in the identified studies, we performed an international, multicenter, patient-level analysis to evaluate the differences in: 1) short- and intermediate-term mortality; 2) length of hospital stay and readmission for HF exacerbation; and 3) comparison of clinical outcomes following TMVr stratified by device success (11).

SEE PAGE 12

METHODS

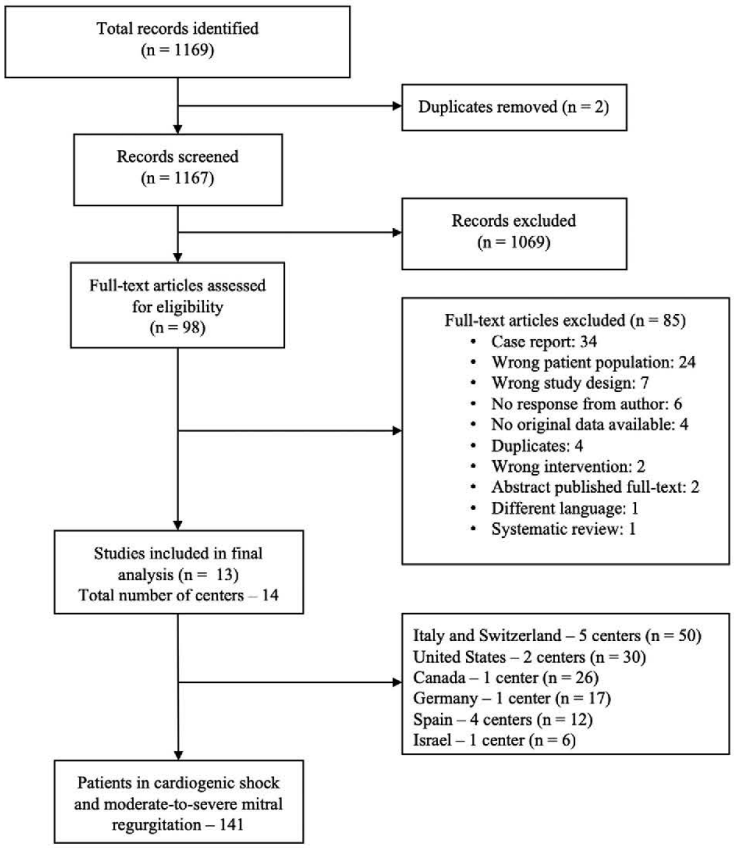
The protocol was registered on September 2019 (PROSPERO: CRD42020151837) and was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses. Each center's research and ethics committee approved its respective registry (7,8,10,12–23), and anonymized data were analyzed at the University of Ottawa Heart Institute.

SEARCH STRATEGY. The search for published studies was guided by a medical librarian using a combination of key terms and index headings such as "MitraClip", "(percutaneous or transcutaneous or transcatheter or catheter-based or endovascular or trans-septal) and (MV repair or "edge-to-edge technique" or Alfieri technique or double-orifice technique)", and "cardiogenic shock" (Supplemental Table 1 for the full search strategy). The final search was conducted on September 13, 2019, in Embase and Embase Classic (1947 to present), Ovid MEDLINE (1946 to present), and the Cochrane Central Register

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The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

Manuscript received June 25, 2020; revised manuscript received August 12, 2020, accepted August 25, 2020.

FIGURE 1 Flow Diagram of Search for Published Studies and Selection for Patient Identification



of Controlled Trials. Search results were exported to Endnote X9 (Thomson Reuters, New York, New York), and duplicates were removed using the program's duplicate identification feature.

Titles and abstracts were screened by two independent reviewers using Covidence (Melbourne, Australia). Eligible studies from screening were reviewed in full by two independent reviewers and were assessed for both inclusion and exclusion criteria. Corresponding investigators from the identified studies were contacted for the patient-level analysis.

INCLUSION AND EXCLUSION CRITERIA. We included case series, prospective, and retrospective studies reporting the use of the MitraClip or TMVr in critically ill patients (>18 years of age) defined as Society for Cardiovascular Angiography and Interventions (SCAI) stage B to E cardiogenic shock (24) or requiring inotrope, ventilator, or MCS support. We excluded studies in children (<18 years of age), studies written in languages other than English, reviews, case reports, and animal studies. We also excluded studies that did not clearly define critically ill patients meeting the aforementioned criteria.

DATA EXTRACTION AND QUALITY ASSESSMENT. All corresponding investigators with the appropriate patient populations were contacted to obtain patient-level information. Each center's ethics committees approved its respective registry, and anonymized data was transferred to the University of Ottawa Heart Institute for collation and analysis.

TABLE 1 Baseline Characteristics (n = 141)

Age, yrs	68.9 ± 12.1
Male	78 (55.3)
BMI, kg/m^2 (n – 112)	25.7 ± 5.1
BSA, m^2 (n – 112)	1.9 ± 0.3
Coexisting conditions	
Hypertension	85 (60.3)
Dyslipidemia	76 (53.9)
Diabetes	57 (40.4)
Current smoker	46 (32.6)
Atrial fibrillation	63 (44.7)
$CHADS_2$ score >2	79 (56.0)
Anemia	47 (33.3)
Related to HF	
History of HF	115 (81.6)
NYHA functional class (n – 135)	
II	5 (3.7)
III	32 (23.7)
IV	98 (72.6)
Hospitalization for HF within the previous year	92 (65.2)
Permanent pacemaker	46 (32.6)
NT-proBNP, pg/ml	6636.7 ± 8479.3
Related to CKD	
eGFR <60 ml/min	56 (39.7)
CRRT	6 (4.3)
Previous history	
Coronary artery disease	86 (61.0)
Percutaneous coronary intervention	62 (44.0)
PCI within the past 30 days	34 (24.1)
Myocardial infarction	76 (53.9)
Coronary artery bypass grafting	24 (17.0)
Cerebrovascular accident	14 (9.9)
STS risk score (n – 117)	16.1 ± 16.6
INTERMACS score (n – 88)	3.1 ± 1.0
SCAI cardiogenic shock class	
B	18 (12.8)
C	71 (50.4)
D	42 (29.8)
E	10 (7.1)
Lactate, mmol/l	3.5 ± 2.8
Intubated	51 (36.2)
Mechanical circulatory support	71 (50.4)
Mechanism of MR	
Functional	106 (75.2)
Degenerative	33 (23.4)
Both	2 (1.4)
Medication pre-MitraClip	
ASA	65 (46.1)
$P2Y_{12}$ inhibitor	28 (19.9)
Anticoagulation	47 (33.3)
ACE inhibitor/ARB	49 (34.8)
Beta-blocker	77 (54.6)
Calcium-channel blocker	7 (5.0)
Mineralocorticoid receptor antagonist	46 (32.6)
Diuretic agent	119 (84.4)

Continued on the next page

The following characteristics were extracted for each patient: demographics and risk factors; MR mechanism; right heart catheterization measurements; presence of mechanical ventilator, inotrope, vasopressor, or MCS support prior to TMVr; echocardiographic results pre- and post-TMVr; and procedural data (see Supplemental Table 2 for data template). Outcomes including mortality, HF hospitalization cerebrovascular accident, length of hospital stay, and discontinuation of pharmacological support or MCS was collected at follow-up.

STATISTICAL ANALYSIS. Continuous variables are reported as mean ± SD or median (interquartile range [IQR]), and categorical variables are reported as proportions. Normally distributed continuous variables were compared using Student's *t*-test or paired *t*-test when applicable. Chi-square, Fisher exact, or McNemar tests were used to compare categorical variables.

The primary endpoint was in-hospital mortality following TMVr. Secondary outcomes were 90-day mortality, HF hospitalization and the combined event rate of 90-day mortality and HF hospitalization after stratification by device success (11). Acceptable device success was defined as a reduction of post-procedural MR by ≥1 grade prior to the procedure to an absolute level ≤2+ (11). The overall incidence of mortality and hospitalization for HF was generated using the Kaplan-Meier method. Changes in pre- and post-procedural MR grade were compared using the McNemar test after dichotomizing MR grades 1 and 2 and grades 3 and 4. Furthermore, pre- and post-procedural left ventricular ejection fraction (LVEF) was compared using a paired *t*-test, and simple linear regression was performed to evaluate the association between baseline and follow-up LVEF.

Device success and in-hospital and 90-day mortality and HF hospitalization event rates were estimated using the Kaplan-Meier method. Furthermore, hazard ratios (HRs) and two-sided 95% confidence intervals (CIs) with and without adjusting for age and sex to determine the association between device success and mortality or HF hospitalization were generated using a Cox proportional hazards model. Post hoc analysis was performed to evaluate differences in MR etiology (primary vs. secondary), reduced LVEF (<35%), acute versus chronic MR, and SCAI cardiogenic shock class to in-hospital mortality by the Cox proportional hazards model. All statistical analyses were conducted using SAS version 9.4 (SAS Institute, Cary, North Carolina). Statistical significance was defined as $p < 0.05$. All figures were generated using GraphPad Prism version 8 (GraphPad Software, La Jolla, California).

RESULTS

A total of 1,167 titles and abstracts were screened, and 98 full-text papers were reviewed for eligibility

TABLE 1 Continued

Hemodynamics pre-MitraClip	
Inotrope	111 (78.7)
Vasopressor	23 (16.3)
Heart rate, beats/min (n – 74)	86.3 ± 17.4
Systolic blood pressure, mm Hg (n – 62)	100.3 ± 18.5
Diastolic blood pressure, mm Hg (n – 62)	58.3 ± 12.4
Mean arterial pressure, mm Hg (n – 68)	74.4 ± 12.7
Pulmonary capillary wedge pressure, mm Hg (n – 55)	25.7 ± 8.3
Right atrial pressure, mm Hg (n – 44)	14.4 ± 7.7
Pulmonary artery systolic pressure, mm Hg (n – 58)	54.6 ± 15.5
Pulmonary artery diastolic pressure, mm Hg (n – 47)	25.9 ± 8.1
Pulmonary artery mean pressure, mm Hg (n – 56)	36.9 ± 10.1
Cardiac output, l/min (n – 46)	3.7 ± 1.4
Cardiac index, l/min/m^2 (n – 55)	1.9 ± 0.7

Values are mean ± SD or n (%).

ACE – angiotensin-converting enzyme; ARB – angiotensin receptor blocker; ASA – acetylsalicylic acid; BMI – body mass index; BSA – body surface area; CKD – chronic kidney disease; CRRT – continuous renal replacement therapy; eGFR – estimated glomerular filtration rate; HF – heart failure; MR – mitral regurgitation; NT-proBNP – N-terminal pro-brain natriuretic peptide; NYHA – New York Heart Association; PCI – percutaneous coronary intervention; SCAI – Society for Cardiovascular Angiography and Interventions; STS – Society of Thoracic Surgeons.

(Figure 1). Eighty-five papers were excluded because they were case reports, the wrong patient population or intervention was studied, or there was a lack of response from the corresponding investigator and missing the outcome of interest. Ultimately, we identified 13 studies from 14 institutions involving a total of 141 patients in cardiogenic shock with moderate to severe MR (see Supplemental Table 3 for institutional-level contribution) (7,8,10,12–23).

PATIENT POPULATION. Between January 2011 and February 2019, 141 patients with cardiogenic shock and moderate to severe or severe MR who were deemed at prohibitive risk for surgical intervention underwent TMVr (Table 1) (24). The cohort's baseline characteristics reflected the critical nature of the patients, with a mean age of 68.9 years, 72.6% with baseline New York Heart Association functional class IV symptoms, 78.7% inotrope dependent, and 50.4% dependent on MCS despite medical optimization. The clinical status was reflected in the mean Society of Thoracic Surgeons risk score of 16.1 and INTERMACS score of 3.1. The mean LVEF was 33.8 ± 14.0%, and MR grade was 3+ in 15 patients (10.6%) and 4+ in 126 patients (89.4%). Furthermore, 71 patients (50.4%) had been classified with SCAI stage C cardiogenic shock, 42 patients (29.8%) with stage D cardiogenic shock, and 10 patients (7.1%) with stage E cardiogenic shock. Finally, the mean cardiac index of the entire cohort was 1.9 ± 0.7 l/min/m^2 (n = 55), the mean pulmonary capillary wedge pressure was 25.7 ± 8.3 mm Hg (n = 55), and the mean pulmonary artery pressure was 36.9 ± 10.1 mm Hg (n = 56).

TABLE 2 Procedural Characteristics

Number of MitraClips used (n –122)	1.0 (1.0-2.0)
Post-MitraClip trans-mitral gradient, mm Hg (n – 57)	4.4 ± 2.8
Procedural time, min (n – 75)	130.6 ± 79.7
Pre-procedural MR	
3	15 (10.6)
4	126 (89.4)
Post-procedural MR	
1 or 2	125 (88.7)
3 or 4	16 (11.3)

Values are median (interquartile range), mean ± SD, or n (%).
MR – mitral regurgitation.

All patients underwent TMVr, with a median time from admission of 11 days (IQR: 4 to 23 days), a median of 1.0 (IQR: 1.0 to 2.0) MitraClips implanted, and a mean procedural time of 130.6 ± 79.7 min (Table 2). Following TMVr, MR grade was 1 or 2+ in 125 patients (88.7%) and 3 or 4+ in 16 patients (11.3%). No procedural complications occurred during TMVr in this cohort.

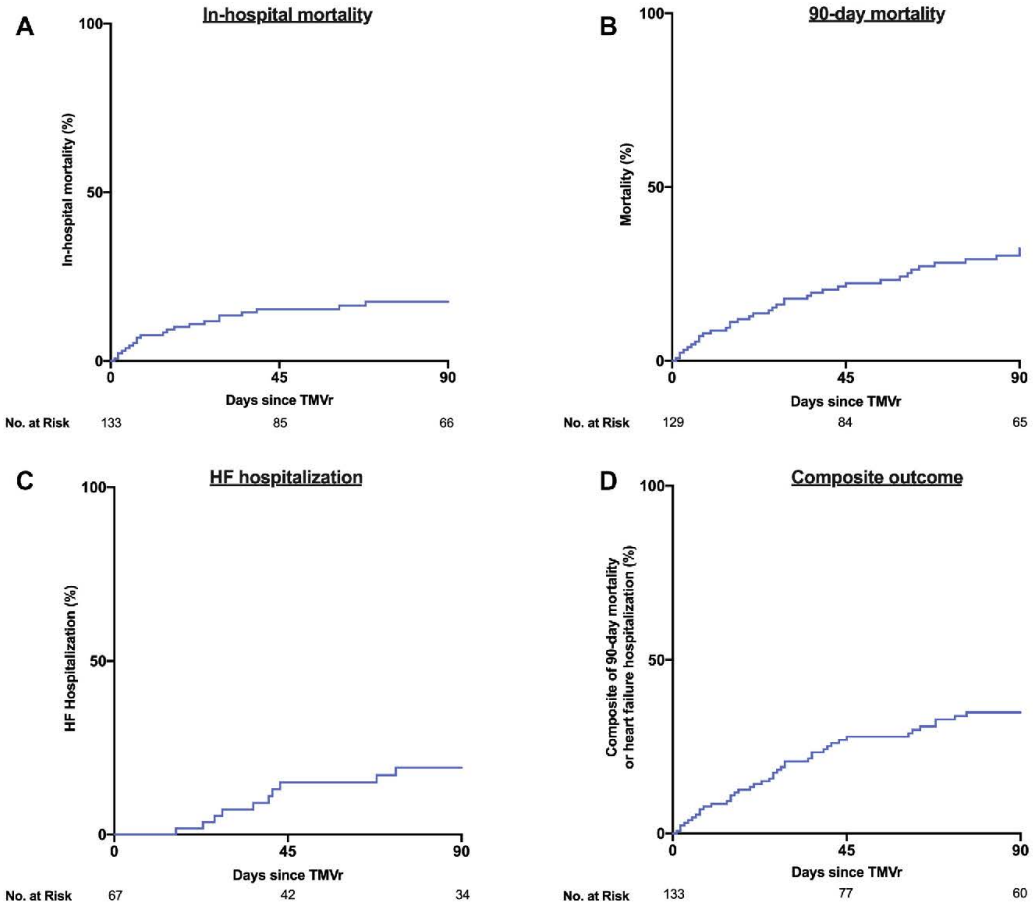
CLINICAL AND ECHOCARDIOGRAPHIC OUTCOMES. Follow-up was complete in all patients, with a median duration of 93 days (IQR: 34.5 to 406.5 days). In-hospital death occurred in 22 patients (15.6%), 90-day mortality in 38 patients (29.5%), and 1-year mortality in 55 patients (42.6%) (Table 3, Figures 2A and 2B). Patients who died were older and more likely to have anemia, higher lactate levels, and higher Society of Thoracic Surgeons risk scores (Supplemental Table 4). The median length of stay in the hospital following the procedure was 10.0 days (IQR: 6.0 to 20.0 days), with a median duration of stay in the intensive care unit of 3.0 days post-implantation (IQR: 1.0 to 8.0 days). Hospital admission for HF following TMVr

TABLE 3 Clinical Outcomes

In-hospital	
Mortality (n – 141)	22 (15.6)
Duration of hospital stay, days (n – 138)	10.0 (6.0-20.0)
Duration of ICU stay, days (n – 103)	3.0 (1.0-8.0)
Inotrope dependence prior to discharge (n – 58)	13 (9.2)
Time to inotrope discontinuation, days	1.0 (0.0-6.0)
Follow-up	
90-day mortality (n – 129)	38 (29.5)
1-yr mortality (n – 129)	55 (42.6)
CHF admission (n – 141)	26 (18.4)
90-day composite outcome (n – 133)	41 (30.8)

Values are median (interquartile range) or n (%).
CHF – congestive heart failure; ICU – intensive care unit.

FIGURE 2 Kaplan-Meier Curves Following TMVr in Patients With Cardiogenic Shock



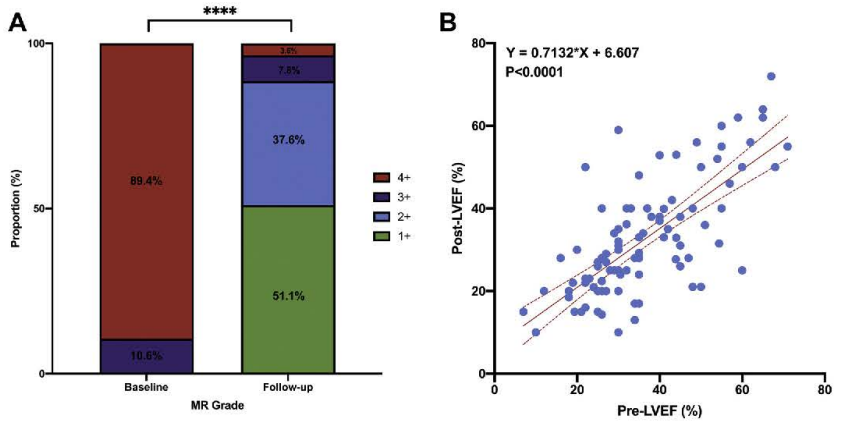
(A) In-hospital mortality occurred in 22 patients in cardiogenic shock (15.6%) following transcatheter mitral valve repair (TMVr). **(B)** 90-day mortality occurred in 38 patients in cardiogenic shock (29.5%) following TMVr. **(C)** Hospitalization for heart failure occurred in 26 patients in cardiogenic shock (18.4%) following TMVr. **(D)** The composite of 90-day mortality and heart failure (HF) hospitalization occurred 41 patients (30.8%) following TMVr.

was observed in 26 patients (18.4%), with a median of 73 days (IQR: 26 to 546 days) (Figure 2C). The composite of 90-day mortality and HF hospitalization occurred in 41 patients (30.8%) (Figure 2D).

All patients underwent post-procedural echocardiography, with a median time of 36 days (IQR: 4 to 399 days). MR grade pre- and post-TMVr was compared in a paired analysis demonstrating a significant reduction in MR grade ($p < 0.0001$) (Figure 3A). Pre- and post-procedural LVEF was reduced following TMVr, with a mean difference of $3.7 \pm 10.4\%$ ($p = 0.0006$) (Supplemental Table 5, Figure 3B).

To assess the impact of MR reduction on survival, patients with device success were compared to those without device success. Acceptable device success was achieved in 125 patients (88.7%), defined as post-procedural change MR by ≥1 grade and an absolute grade of ≤2+ on follow-up echocardiography, and 16 patients (11.3%) failed to achieve device success,

FIGURE 3 Quantitative Improvement in MR and Reduction in LVEF in Patients in Cardiogenic Shock Undergoing TMVr



(A) Mitral regurgitation (MR) severity improved immediately and at 1-year follow-up after transcatheter mitral valve repair (TMVr) with 88.7% device success. **(B)** Comparison of pre- and post-procedural left ventricular ejection fraction (LVEF) demonstrates a mean reduction of 4.0 ± 10.8% following TMVr. Changes in MR grade were compared using the McNemar test. Line of best fit was generated by a linear regression model and compared using Spearman's correlation test. ****$p < 0.0001$.

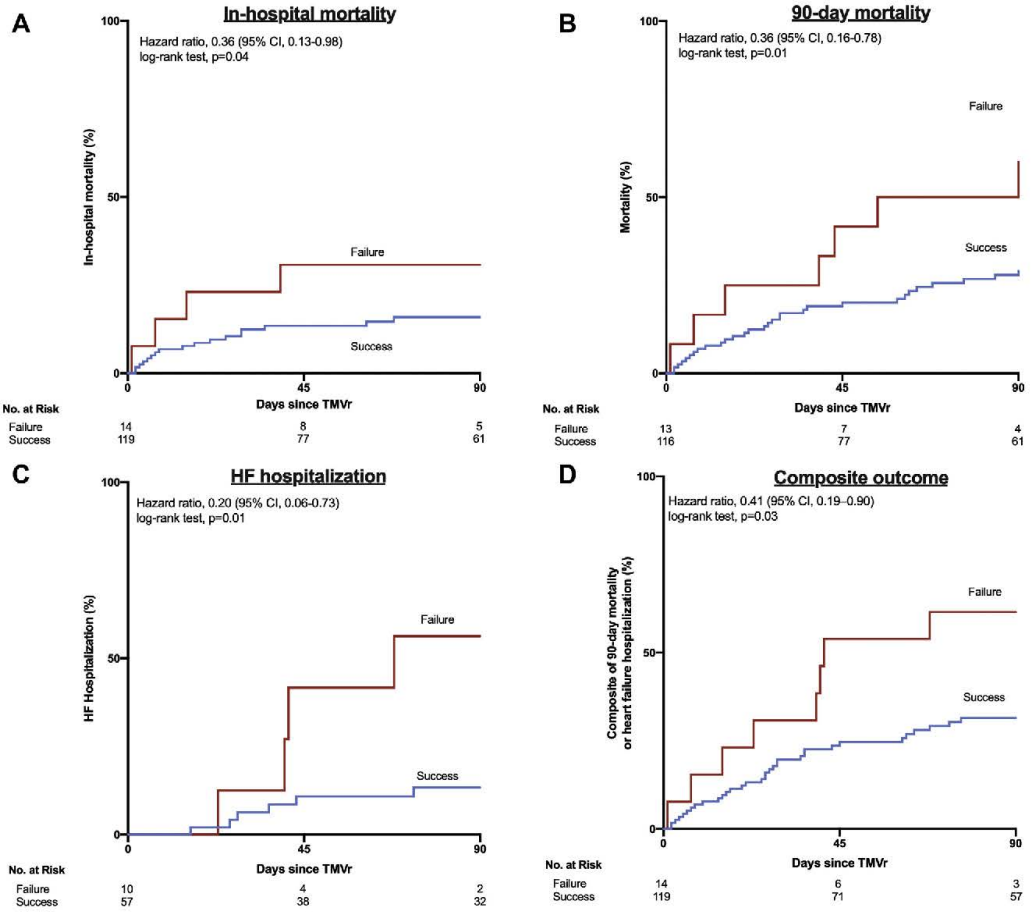
with no differences in baseline characteristics (Supplemental Table 6). Specifically, in patients in whom MR reduction was successful, there was lower in-hospital mortality (HR: 0.36; 95% CI: 0.13 to 0.98; p = 0.04) (**Figure 4A**, Supplemental Table 7). Device success was also associated with reduced rates of 90-day mortality (HR: 0.36; 95% CI: 0.16 to 0.78; p = 0.01) (**Figure 4B**) and 1-year mortality (HR: 0.46; 95% CI: 0.22 to 0.94; p = 0.03), and the association remained after adjusting for age and sex (Supplemental Table 7). Finally, device success was associated with lower rates of 90-day HF hospitalization following the procedure (HR: 0.20; 95% CI: 0.06 to 0.73; p = 0.01) (**Figure 4C**) and the composite of 90-day mortality and HF hospitalization (HR: 0.41; 95% CI: 0.19 to 0.90; p = 0.03) (**Figure 4D**). In-hospital mortality stratified by important subgroups, including baseline SCAI class, baseline LVEF, primary versus secondary, and acute versus chronic MR, are presented in Supplemental Figure 1.

DISCUSSION

In the present study, TMVr using the MitraClip system appears to be a viable therapeutic salvage strategy, demonstrating procedural safety and efficacy in MR reduction in patients with significant MR and cardiogenic shock. Although heterogenous in MR etiology, successful device implantation was associated with decreased risk for mortality over the first year, supporting the hypothesis that MR reduction in the acute phase of shock may represent a therapeutic target addressable using a percutaneous approach (**Central Illustration**).

TMVr is known to reduce mortality and HF hospitalization; however, these benefits have been demonstrated only in patients in stable condition receiving guideline-directed medical therapy, excluding those who were at prohibitive surgical risk and in cardiogenic shock (1–3). Patients in cardiogenic shock with severe valvular disease represent a uniquely high-risk cohort with poor outcomes and limited therapies (7,25,26). Historically, in-hospital mortality in patients with severe MR from acute MI approaches 60% and remained at 40% following mitral valve surgery (5). In our cohort of patients with prohibitive surgical risk, we report an in-hospital mortality rate of 15.6%, with improved survival in those with device success. Indeed, in the setting of cardiogenic shock, increasing MR burden is associated with diminished survival, supporting a "dose effect" (27). Although our 1-year mortality in the total cohort was 50% (25,28,29), we further demonstrate that device success reduced 1-year mortality to 39.7% compared with 69.2% in those without device success, reflecting the high risk in baseline without MR treatment. Collectively, these observations suggest that the use of TMVr to reduce MR severity in

FIGURE 4 Kaplan-Meier Curves Following TMVr in Patients With Cardiogenic Shock Stratified by TMVr Device Success in MR

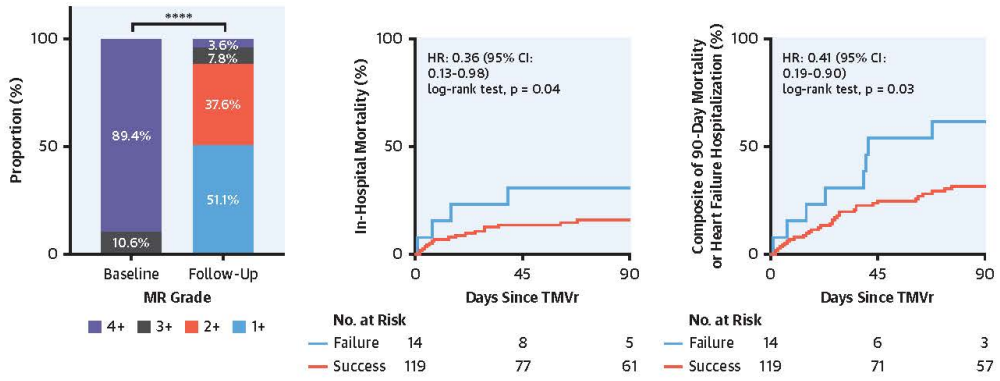


(A) Cumulative incidence of in-hospital mortality among patients who underwent transcatheter mitral valve repair (TMVr). Device success was associated with reduced rates of in-hospital mortality (hazard ratio [HR]: 0.36; 95% confidence interval [CI]: 0.13 to 0.98; p = 0.04). **(B)** Cumulative incidence of mortality at 90-day follow-up among patients who underwent TMVr. Patients with device success had reduced rates of 90-day mortality (HR: 0.36; 95% CI: 0.16 to 0.78; p = 0.01). **(C)** In patients with hospitalization for heart failure (HF) at 90-day follow-up among those who underwent TMVr, those with device success had a lower incidence of hospitalization for heart failure (HR: 0.20; 95% CI: 0.06 to 0.73; p = 0.01). **(D)** In patients with 90-day mortality and HF hospitalization at 90-day follow-up, device success was associated with a reduced cumulative event rate (HR: 0.41; 95% CI: 0.19 to 0.90; p = 0.03). Kaplan-Meier curves were generated and compared using the log-rank test, and HRs were evaluated using the Cox proportional hazards model. P values < 0.05 were considered to indicate statistical significance.

cardiogenic shock may translate to improved clinical outcomes.

Several considerations remain in selecting an optimal management strategy for patients with cardiogenic shock and significant MR. Currently, revascularization remains a cornerstone in the treatment of shock of ischemic etiology, and MCS is being increasingly used up front in the management of these patients (30,31). Although one-half of the patients in our study had temporary MCS, with a majority receiving intra-aortic balloon pump support, all were deemed ineligible for escalation to durable support or transplantation at the time of TMVr (32). In the COAPT study, TMVr was associated with a 50%

CENTRAL ILLUSTRATION **Impact of Successful Transcatheter Mitral Valve Repair on Clinical Outcomes in Patients With Cardiogenic Shock and Mitral Regurgitation**



Jung, R.G. et al. J Am Coll Cardiol Intv. 2021;14(1):1-11.

Cumulative incidence of in-hospital mortality and composite of 90-day mortality and heart failure hospitalization among patients who underwent transcatheter mitral valve repair (TMVr) stratified by residual mitral regurgitation. **** indicates $p < 0.0001$. HR = hazard ratio; MR = mitral regurgitation.

reduction in the need for left ventricular assist device implantation (1). Although patients in the present study were deemed ineligible, in patients with cardiogenic shock and MR who are candidates for durable MCS, consideration for TMVr as either a bridge or adjunctive therapy may be reasonable. Moreover, MCS is reported to carry a 30% risk for complications including hemolysis, infection, and bleeding (30), while TMVr is a transvenous procedure with an exceptional safety profile, even in this high-risk cohort.

STUDY LIMITATIONS. First, the data are observational and are thus susceptible to all biases inherent to this study design.

Second, although reported outcomes following TMVr are encouraging, the lack of a comparison group who did not undergo intervention is notable. However, in the present study, the use of TMVr was a last-line salvage therapy, with ineligible patients typically receiving palliative care and dying in the hospital. Decisions to not treat these patients would reflect differences in baseline characteristics that could not be reasonably adjusted for.

Third, some hemodynamic parameters from right heart catheterization and echocardiographic data were missing from the cohort.

Finally, despite encompassing all available patient-level data on this cohort, the overall size remains small. Thus, although we were able to compare outcomes between patients with and those without device success, more robust adjustment of the data was not possible.

CONCLUSIONS

In patients with cardiogenic shock with moderate to severe MR, TMVr is safe and technically feasible. Dedicated prospective studies are warranted, while TMVr may be considered in select patients pending the outcomes of larger clinical studies.

ACKNOWLEDGMENTS The authors thank all the collaborators and the CAPITAL Research Group for all their support and guidance for the completion of the project.

AUTHOR DISCLOSURES

Dr. Jung was funded by the Vanier CIHR Canada Graduate Scholarship. Dr. Benito-Gonzalez has received grants from Abbott Vascular, outside the summitted work. Dr. Estevez-Loureiro is a consultant for Abbott Vascular; and is a proctor for the MitraClip, outside the submitted work. Dr. Buzzatti has received personal fees from InnovHeart, outside the submitted work. Dr. Hibbert has received funding as a clinical trial investigator from Abbott, Boston Scientific, and Edwards Lifesciences, outside the submitted work. All other authors have reported that they have no relationships relevant to the contents of this paper to disclose.

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PERSPECTIVES

WHAT IS KNOWN? Cardiogenic shock with severe MR is associated with poor outcomes with limited therapeutic options. Moderate or greater MR represents 5% to 10% of patients in cardiogenic shock, and these patients are often at prohibitive risk for surgical intervention and are not candidates for advanced MCS. The role of TMVr as an adjunct therapy in patients with cardiogenic shock remains poorly described.

WHAT IS NEW? The present study demonstrates that successful TMVr can be performed as a salvage therapy in patients with no further options. Successful repair was associated with improved outcomes in this high-risk cohort and presents a novel therapeutic option in select patients.

WHAT IS NEXT? This study demonstrates the potential of TMVr as a salvage therapy, and prospective randomized studies are warranted to assess whether valvular intervention in those with cardiogenic shock and significant MR improves mortality in this high-risk cohort.

REFERENCES

1. Stone GW, Lindenfeld J, Abraham WT, et al. Transcatheter mitral-valve repair in patients with heart failure. N Engl J Med 2018;379:2307–18.

2. Feldman T, Foster E, Glower DD, et al. Percutaneous repair or surgery for mitral regurgitation. N Engl J Med 2011;364:1395–406.

3. Obadia JF, Messika-Zeitoun D, Leurent G, et al. Percutaneous repair or medical treatment for secondary mitral regurgitation. N Engl J Med 2018;379:2297–306.

4. Baumgartner H, Falk V, Bax JJ, et al. 2017 ESC/EACTS guidelines for the management of valvular heart disease. Eur Heart J 2017;38:2739–91.

5. Thompson CR, Buller CE, Sleeper LA, et al. Cardiogenic shock due to acute severe mitral regurgitation complicating acute myocardial infarction: a report from the SHOCK trial registry. J Am Coll Cardiol 2000;36:1104–9.

6. Thiele H, Ohman EM, Desch S, Eitel I, de Waha S. Management of cardiogenic shock. Eur Heart J 2015;36:1223–30.

7. Flint K, Brieke A, Wiktor D, Carroll J. Percutaneous edge-to-edge mitral valve repair may rescue select patients in cardiogenic shock: findings from a single center case series. Catheter Cardiovasc Interv 2019;94:E82–7.

8. Chan V, Messika-Zeitoun D, Labinaz M, et al. Percutaneous mitral repair as salvage therapy in patients with mitral regurgitation and refractory cardiogenic shock. Circ Cardiovasc Interv 2019;12:e008435.

9. Pleger ST, Chorianopoulos E, Krumsdorf U, Katus HA, Bekeredjian R. Percutaneous edge-to-edge repair of mitral regurgitation as a bail-out strategy in critically ill patients. J Invas Cardiol 2013;25:69–72.

10. Turyan Medvedovsky A, Tonchev I, Tahiroglu I, et al. MitraClip therapy in critically ill patients with severe functional mitral regurgitation and refractory heart failure. Struct Heart 2019;3:296–301.

11. Stone GW, Adams DH, Abraham WT, et al. Clinical trial design principles and endpoint definitions for transcatheter mitral valve repair and replacement: part 2: endpoint definitions: a consensus document from the Mitral Valve Academic Research Consortium. J Am Coll Cardiol 2015;66:308–21.

12. Adamo M, Barbanti M, Curello S, et al. Effectiveness of MitraClip therapy in patients with refractory heart failure. J Interv Cardiol 2015;28:61–8.

13. Adamo M, Curello S, Chiari E, et al. Percutaneous edge-to-edge mitral valve repair for the treatment of acute mitral regurgitation complicating myocardial infarction: A single centre experience. J Int Cardiol 2017;234:53–7.

14. Di Prima AL, Covello DR, Franco A, et al. Do patients undergoing MitraClip implantation require routine ICU admission? J Cardiothorac Vasc Anesth 2014;28:1479–83.

15. Eliaz R, Turyan A, Beeri R, Shuvy M. Utilization of intra-aortic balloon pump to allow MitraClip procedure in patients with non-coapting mitral valve leaflets: a case series. Eur Heart J Case Rep 2019;3:ytz045.

16. Estevez-Loureiro R, Arzamendi D, Freixa X, et al. Percutaneous mitral valve repair for acute mitral regurgitation after an acute myocardial infarction. J Am Coll Cardiol 2015;66:91–2.

17. Kesler M, Seeger J, Wohrle J, Rottbauer W, Markovic S. Intra-aortic balloon counterpulsation pump in heart failure patients during MitraClip implantation–a propensity-score matched analysis. Catheter Cardiovasc Interv 2018;92:1433–8.

18. Samuel Del Castillo Garcia D, Carlos Minguito Carazo D, Tomas Benito Gonzalez D, et al. Outcomes following urgent percutaneous mitral valve repair: a single center observational study. Eur Heart J Acute Cardiovasc Care 2019;8 suppl 1:15–7.

19. Taramasso M, Denti P, Cioni M, et al. Percutaneous mitral valve repair in high-risk patients: Single center experience. EuroIntervention, 2012. N:N188.

20. Berardini A, Biagini E, Saia F, et al. Percutaneous mitral valve repair: the last chance for symptoms improvement in advanced refractory chronic heart failure? J Int Cardiol 2017;228:191–7.

21. Buzzatti N, Taramasso M, Cioni M, et al. MitraClip therapy in idiopathic vs ischemic dilated cardiomyopathy, a single center experience. J Am Coll Cardiol 2013;1:B213.

22. Haberman D, Taramasso M, Czarnecki A, et al. Salvage MitraClip in severe secondary mitral regurgitation complicating acute myocardial infarction: data from a multicentre international study. Eur J Heart Fail 2019;21:1161–4.

23. Kovach CP, Bell S, Kataruka A, Reisman M, Don C. Outcomes of urgent/emergent transcatheter mitral valve repair (MitraClip): a single center experience. Catheter Cardiovasc Interv. 2020 Jun 26 [E-pub ahead of print].

24. Baran DA, Grines CL, Bailey S, et al. SCAI clinical expert consensus statement on the classification of cardiogenic shock: this document was endorsed by the American College of Cardiology (ACC), the American Heart Association (AHA), the Society of Critical Care Medicine (SCCM), and the Society of Thoracic Surgeons (STS) in April 2019. Catheter Cardiovasc Interv 2019;94:29–37.

25. Thiele H, Akin I, Sandri M, et al. PCI strategies in patients with acute myocardial infarction and cardiogenic shock. N Engl J Med 2017;377:2419–32.

26. Sv Diepen, Katz JN, Albert NM, et al. Contemporary management of cardiogenic shock: a scientific statement from the American Heart Association. Circulation 2017;136:e232–68.

27. Picard Michael H, Davidoff R, Sleeper Lynn A, et al. Echocardiographic predictors of survival and

response to early revascularization in cardiogenic shock. Circulation 2003;107:279–84.

28. Hochman JS, Sleeper LA, Webb JG, et al., for the SHOCK Investigators. Early revascularization in acute myocardial infarction complicated by cardiogenic shock. N Engl J Med 1999;341:625–34.

29. Ouweneel DM, Eriksen E, Sjauw KD, et al. Percutaneous mechanical circulatory support versus intra-aortic balloon pump in cardiogenic shock after acute myocardial infarction. J Am Coll Cardiol 2017;69:278–87.

30. Russo JJ, Aleksova N, Pitcher I, et al. Left ventricular unloading during extracorporeal membrane oxygenation in patients with cardiogenic shock. J Am Coll Cardiol 2019;73:654–62.

31. Tehrani BN, Truesdell AG, Sherwood MW, et al. Standardized team-based care for cardiogenic shock. J Am Coll Cardiol 2019;73:1659–69.

32. Estevez-Loureiro R, Adamo M, Arzamendi D, et al. Transcatheter mitral valve repair in patients with acute myocardial infarction: insights from the European Registry of MitraClip in Acute Mitral Regurgitation following an acute myocardial infarction (EREMMI). EuroIntervention 2020;15:1248–50.

KEY WORDS cardiogenic shock, mitral regurgitation, transcatheter mitral valve repair

APPENDIX For supplemental tables and a figure, please see the online version of this paper.



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Appendix E

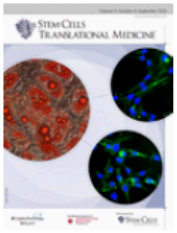
The secretome of liver X receptor agonist treated early outgrowth cells decreases atherosclerosis in Ldlr-/- mice

Citation information:

Rasheed A, Shawky S, Tsai R, **Jung RG**, Simard T, Saikali M, Hibbert B, Rayner KJ, Cummins CL. The secretome of liver X receptor agonist treated early outgrowth cells decreases atherosclerosis in Ldlr-/- mice. **(2020)**. *Stem Cell Translational Medicine*.

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The secretome of liver X receptor agonist-treated early outgrowth cells decreases atherosclerosis in Ldlr−/− mice

Author: Carolyn L. Cummins, Katey J. Rayner, Benjamin Hibbert, et al

Publication: STEM CELLS Translational Medicine

Publisher: John Wiley and Sons

Date: Nov 24, 2020

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Received: 16 November 2019 | Accepted: 26 September 2020
DOI: 10.1002/sctm.19-0390

TISSUE-SPECIFIC PROGENITOR AND STEM CELLS

STEM CELLS TRANSLATIONAL MEDICINE

The secretome of liver X receptor agonist-treated early outgrowth cells decreases atherosclerosis in *Ldlr*−/− mice

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Funding information
Dean's Fund of the Leslie Dan Faculty of Pharmacy; Ontario Graduate Scholarship; Heart and Stroke Foundation of Canada, Grant/Award Numbers: G-18-0022364, G-13-0002612

Abstract

Endothelial progenitor cells (EPCs) promote the maintenance of the endothelium by secreting vasoreparative factors. A population of EPCs known as early outgrowth cells (EOCs) is being investigated as novel cell-based therapies for the treatment of cardiovascular disease. We previously demonstrated that the absence of liver X receptors (LXRs) is detrimental to the formation and function of EOCs under hypercholesterolemic conditions. Here, we investigate whether LXR activation in EOCs is beneficial for the treatment of atherosclerosis. EOCs were differentiated from the bone marrow of wild-type (WT) and LXR-knockout (*Lxrαβ*−/−) mice in the presence of vehicle or LXR agonist (GW3965). WT EOCs treated with GW3965 throughout differentiation showed reduced mRNA expression of endothelial lineage markers (*Cd144*, *Vegfr2*) compared with WT vehicle and *Lxrαβ*−/− EOCs. GW3965-treated EOCs produced secreted factors that reduced monocyte adhesion to activated endothelial cells in culture. When injected into atherosclerosis-prone *Ldlr*−/− mice, GW3965-treated EOCs, or their corresponding conditioned media (CM) were both able to reduce aortic sinus plaque burden compared with controls. Furthermore, when human EOCs (obtained from patients with established CAD) were treated with GW3965 and the CM applied to endothelial cells, monocyte adhesion was decreased, indicating that our results in mice could be translated to patients. Ex vivo LXR agonist treatment of EOCs therefore produces a secretome that decreases early atherosclerosis in *Ldlr*−/− mice, and additionally, CM from human EOCs significantly inhibits monocyte to endothelial adhesion. Thus, active factor(s) within the GW3965-treated EOC secretome may have the potential to be useful for the treatment of atherosclerosis.

KEYWORDS

atherosclerosis, autologous cell therapy, early outgrowth cells, liver X receptor, secreted factors

Abbreviations: CAD, coronary artery disease; CM, conditioned media; EndMT, endothelial-to-mesenchymal transition; EOC, early outgrowth cell; EPC, endothelial progenitor cell; HUVEC, human umbilical vein endothelial cell; LXR, liver X receptor; WT, wild type.

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1 | INTRODUCTION

Cardiovascular disease remains the leading cause of death worldwide.[1] Atherosclerosis is a vascular complication arising from cardiovascular disease and is typically diagnosed in its later stages, after established lipid-laden plaques have deposited in the aorta. Plaque development is initiated by coordinate dysfunction to various vessel wall cell types, including endothelial and vascular smooth muscle cells, as well as hematopoietic cell types.[2] Prior to plaque deposition, the aortic endothelium undergoes pathological activation resulting from systemic inflammation and elevations in plasma lipoproteins. During endothelial activation, selectins and adhesion molecules are upregulated, which facilitate the binding of monocytes to the endothelium. Adherent monocytes then traverse the endothelium to the intima, and differentiate into macrophages which engulf modified lipoproteins to promote the plaque development characteristic of established atherosclerosis.[3]

The liver X receptors, LXRα (*Nr1h3*) and LXRβ (*Nr1h2*), belong to the nuclear receptor superfamily of transcription factors.[4,5] Activation of the LXRs induces the gene expression of the cholesterol efflux transporters, *Abca1* and *Abcg1*; and represses pro-inflammatory gene expression, including *Mcp-1*, *Tnfα*, and *Il-1β*.[6-8] These roles of LXRs have been particularly well characterized in macrophages, in part through bone marrow transplant experiments that support an anti-atherogenic role for LXRs.[9-14] However, recent bone marrow transplantation studies using bone marrow deficient in LXR target genes have demonstrated that LXRs may elicit their anti-atherogenic roles in other bone marrow-derived cell types apart from monocytes/macrophages.[14-17] In the bone marrow, hematopoietic stem cells (HSCs) can differentiate into myeloid progenitors, which produce the monocytes/macrophages known to contribute directly to plaque progression.[3,18] HSCs, however, can also differentiate into other cell types including endothelial progenitor cells (EPCs).[19,20]

EPCs were initially described in a report by Asahara et al in 1997 as peripheral blood cells that differentiate into endothelial-like cells and contribute to vascular repair by direct incorporation.[19] Many studies have since demonstrated that patients with diabetes or cardiovascular disease have dysfunction in EPC numbers and function.[21-23] Over the past 20 years, EPC function has largely been characterized using ex vivo cultures, which yield two cell populations: early outgrowth cells (EOCs; 7-10 days in culture) and late outgrowth endothelial cells (14-21 days in culture). EOCs participate in endothelial repair through the secretion of factors, whereas late outgrowth cells differentiate to endothelial-like cells and promote vascular homeostasis through direct incorporation.[24-27] Consistent with observations made by Asahara and colleagues, EPC studies were initially focused on their endothelial-like phenotype and how they facilitate neovascularization by incorporation into the damaged vessel.[28-33] However, studies have since shown inconsistent engraftment in vivo, suggesting that endothelial repair by EPCs occurs via an indirect mechanism.[26,34-37] Given these discrepancies, attention has shifted to the EOC subpopulation that supports endothelial repair by the secretion of vasoreparative factors, which has spurred a new generation of clinical trials[38,39] and applications in regenerative medicine, such as those reviewed by Chong et al.[40]

Significance statement

Patient-derived early outgrowth cells (EOCs) are being investigated as a potential treatment for vascular diseases, including pulmonary hypertension and myocardial infarction. The data of this study demonstrate that liver X receptor (LXR) activation of mouse EOCs causes the secretion of factors that reduce binding of inflammatory cells to endothelial cells and ultimately decrease the development of atherosclerosis. This study also shows that LXR agonist-treated EOCs derived from human coronary artery disease patients recapitulate these results in cell culture. Therefore, this study suggests that treatment with autologous LXR agonist-treated EOCs and/or the isolation of the beneficial factors that are secreted may help decrease the progression of atherosclerosis.

Using *Lxrαβ−/−* double knockout mice fed a hypercholesterolemic diet, we previously demonstrated that LXRs are essential for preventing cholesterol-induced defects in EOCs.[41] Elevated cholesterol content, only in the absence of LXRs, altered EOC differentiation and the resulting secretome, which increased monocyte adhesion to treated endothelial cells in vitro. As a result of these studies, here we set out to determine: (a) whether pharmacologic activation of LXRs could beneficially influence EOC differentiation and secretome function and (b) if ex vivo pharmacological activation of LXRs in EOCs could prevent the development of atherosclerosis in vivo. Herein, we present data that show pharmacological activation of LXRs reduces the expression of endothelial lineage markers in wild-type (WT) EOCs, and alters the EOC secretome in a manner that inhibits monocyte adhesion to treated endothelial cells. Tail vein administration of either LXR agonist-treated WT EOCs or their conditioned media (CM) to *Ldlr−/−* mice attenuated lesion development in the aortic sinus compared to their respective controls. Furthermore, we demonstrate the translational potential of LXR agonist-treatment of EOCs using EOCs derived from patients with established coronary artery disease (CAD). The studies presented here suggest that activation of LXRs in EOCs ex vivo may therefore provide a new avenue for therapeutic intervention in atherosclerosis.

2 | MATERIALS AND METHODS

2.1 | Mice

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Toronto. WT and LXR double knockout (*Lxrαβ−/−*) male mice were backcrossed more than 10 generations on a C57Bl/6 background and maintained on a chow diet

(#2016 Harlan Teklad, Mississauga, Ontario, Canada). *Ldlr*-knockout male mice (*Ldlr*−/−; B6.129S7-Ldlr^{tm1Her}/J, Stock #002207) on a C57Bl6/J background were purchased from Jackson laboratories (Bar Harbor, Maine). All mice were housed in a temperature and light-controlled environment. Mice were sacrificed between 9 AM and 11 AM by cervical dislocation or exsanguination under isoflurane anesthesia.

2.2 | Bone marrow harvesting and EOC culture

EOCs were cultured from the bone marrow of the tibiae and femurs of WT and *Lxrαβ*−/− mice. The bone marrow cells were seeded on human-fibronectin coated (Sigma-Aldrich, Oakville, Ontario, Canada) tissue culture dishes and differentiated for 7 days to EOCs in endothelial basal media supplemented with growth factors/cytokines (EGM-2 Bullet Kit; Lonza, Walkersville, Maryland) at 37°C with 5% CO_2. The media was changed every other day. EOCs were cultured in the presence of vehicle (ethanol) or LXR agonist (1 μM GW3965; Tocris, Minneapolis, Minnesota) throughout the 7-day differentiation protocol.

2.3 | RNA isolation, cDNA synthesis, and real-time QPCR

RNA isolation, cDNA synthesis, and QPCR were performed as previously described.[41] Primer sequences are listed in Supporting Information Table S1 (mouse) and Supporting Information Table S2 (human).

2.4 | CAD patient recruitment

Patients who underwent coronary angiography gave written informed consent for blood collection. This study was approved by the Ottawa Health Science Network Research Ethics Board (OHSN-REB, Protocol #: 20160516-01H). This study conforms to the 1975 Declaration of Helsinki for the use of human blood. The University of Ottawa Heart Institute serves 1.2 million people with all cardiac catheterization procedures prospectively indexed in the Cardiovascular and Percutaneous Clinical Trials (CAPITAL) revascularization registry along with baseline investigations and medications.[42,43] From August 2018 to March 2019, blood samples were collected from 21 patients in EDTA tubes (Becton Dickinson, Franklin Lakes, New Jersey) at the time of coronary angiography. CAD was confirmed by coronary angiography and defined as an epicardial stenosis ≥50%. The presence of type 2 diabetes was defined as hemoglobin A1c (HbA1c) levels ≥6.5% or a prior diagnosis or medical therapy for diabetes. Patient characteristics are provided in Table 1.

2.5 | Human EOC culture

Peripheral blood mononuclear cells were isolated by density centrifugation using Ficoll-Paque Plus (GE Healthcare, Marlborough, Massachusetts). The cells were differentiated to EOCs as described above. For each patient, the mononuclear cells were differentiated to EOCs in both vehicle and 1 μM GW3965.

TABLE 1 Characteristics of coronary artery disease patients from which EOCs were derived

	EOC (n = 21)	
	Number or mean ± SD	Proportion (%)
Age, years	68 ± 13	
Sex (male)	15	71.4
HTN	12	57.1
DM		
Type I	1	4.8
Type II - oral agents	4	19.1
Type II - insulin	2	9.5
Smoker		
Never	16	76.2
Remote (quit >1 month ago)	3	14.3
Active	2	9.5
Dyslipidemia	12	57.1
Family history of CAD	3	14.3
ACS	7	33.3
HF/LV dysfunction	2	9.5
Prior history		
MI	4	19.1
PCI	3	14.3
CVA	1	4.8
CABG	1	4.8
Medications		
ASA	20	95.2
P2Y12	18	85.7
Beta blocker	10	47.6
ACEi/ARB	14	66.7
Statin	19	90.5
Biochemistry		
WBC (10^9 cells/L)	7 ± 2	
HGB (g/L)	134 ± 26	
PLT (10^9 cells/L)	225 ± 52	
HbA1c (%) (n = 16)	6.1 ± 0.8	
Cholesterol (mM) (n = 7)	4 ± 1	
LDL (mM) (n = 7)	2 ± 1	
Creatinine (μM)	92 ± 65	

Abbreviations: ACE, angiotensin-converting enzyme; ACS, acute coronary syndrome; ARB, angiotensin receptor blocker; ASA, acetylsalicylic acid; CABG, coronary artery bypass graft; CVA, cerebrovascular accident; DM, diabetes mellitus; HF/LV, heart failure/left ventricular; HGB, hemoglobin; HTN, hypertension; LDL, low-density lipoprotein; MI, myocardial infarction; PCI, percutaneous coronary intervention; PLT, platelet; WBC, white blood cell.

2.6 | CM collection

After 7 days of differentiation, EOCs were washed twice with cold PBS and incubated in factor- and GW3965-free media (EBM-2; Lonza) for 30 minutes at 37°C and 5% CO_2. The media was discarded and the cells were replenished with fresh EBM-2 media. The CM was then collected after 24 hours, centrifuged at 700*g* for 5 minutes to remove any cells, and syringe filtered (0.22 μm). The CM was frozen at −80°C until use. The number of EOCs from each treatment group was unchanged after differentiation (data not shown).

2.7 | Cell lines

Human umbilical vein endothelial cells (HUVEC) and media (EGM-2 Bullet Kit) were purchased from Lonza. THP-1 monocytes were received as a gift from Dr. Myron I. Cybulsky (University of Toronto) and were maintained in RPMI 1640 with L-glutamine (Sigma-Aldrich) supplemented with 10% FBS and 0.05 mM β-mercaptoethanol (Sigma-Aldrich). All cell lines were used between passages 4 and 6.

2.8 | Monocyte-endothelial adhesion assay

HUVECs were seeded in 12-well plates on sterilized circular glass coverslips (Thermo Fisher Scientific, Hampton, New Hampshire) and treated at 60% confluence with 20% (mouse) or 30% (human) EOC CM in EGM-2 media for 20 hours, followed by a 4-hour cotreatment with EOC CM and 10 ng/mL TNFα (Life Technologies). After treatment, HUVECs were coincubated with 10^5 CMFDA (Life Technologies)-labeled THP-1 monocytes per well for 90 minutes. After coculture, the nonadherent THP-1 cells were washed off and the cells were fixed using 4% paraformaldehyde. Coverslips were mounted onto microscope slides using Vectashield (Vector Labs, Burlingame, California) or Dako Fluorescence (Agilent Technologies, Santa Clara, California) and images were acquired on a laser confocal LSM700 or LSM880 microscope operated by Zen software (Zeiss, Toronto, Ontario, Canada).

2.9 | Splenectomy

A cohort of *Ldlr*−/− mice were splenectomized at 4 weeks of age. The abdominal cavity was opened, and the spleen was removed by cauterizing the splenic arteries. The abdominal cavity and skin were closed using 4-0 gauge synthetic absorbable suture. The mice were then treated with ketoprofen (s.i.d.) and buprenorphine (b.i.d.) for 3 days postsurgery and allowed to recover 4 weeks prior to the initiation of treatments and diet.

2.10 | Preparation of EOCs and concentrated CM for in vivo studies

To generate EOCs and their CM, bone marrow from the femurs and tibiae of two mice were seeded in one 100 mm plate. Day 7 EOCs were detached using Accutase Enzyme Detachment Buffer (eBioscience, San Diego, California) washed once, and resuspended in saline for injections. Approximately 10^7 EOCs were collected at day 7, irrespective of ligand treatment. CM was concentrated 10-fold using a <3 kDa Amicon centrifuge filtration column (Millipore, Etobicoke, Ontario, Canada) and dialyzed twice in saline prior to injection.

2.11 | Treatment and sacrifice of Ldlr−/− mice

Starting at 8 weeks of age, *Ldlr*−/− mice were fed a Western style diet (#88137 Harlan Teklad) for 8 weeks. The splenectomized cohort of the *Ldlr*−/− mice were injected via the tail vein with 10^6 EOCs from WT C57Bl/6 mice in 100 μL of saline. The nonsplenectomized *Ldlr*−/− mice received 100 μL of the 10-fold concentrated CM. Treatments were administered every 2 weeks throughout the Western diet feeding period. Complete blood counts were performed on peripheral blood collected at sacrifice on a VetScan HM5 (Abaxis, Union City, California). Whole blood obtained at sacrifice was centrifuged at 500*g* for 20 minutes at 4°C for the separation of plasma, which was aliquoted and frozen at −80°C until analysis. Plasma cholesterol was determined by enzymatic assay using the Cholesterol Infinity Kit (Thermo Fisher Scientific) and IL-6 quantified by ELISA (Thermo Fisher Scientific) as per the manufacturer's recommendations.

2.12 | Histological analysis

Aortic sinuses were cryosectioned using a Leica CM 3050S cryostat (Concord, Ontario, Canada) at a 10 μm thickness and placed on Superfrost Plus microscope slides (Thermo Fisher Scientific). For lesion quantification, the sections were stained with hematoxylin and eosin (Sigma-Aldrich). For immunohistochemical analyses, the sections were incubated with the primary antibodies VCAM1 (CD106, BD Biosciences, 1:10) or CD45 (BD Biosciences, 1:20) overnight at 4°C followed by the biotinylated anti-rat IgG secondary antibody (Vector Laboratories, 1:100). The sections were washed and then incubated with the ABC kit (Vector Laboratories) for 30 minutes following manufacturer's recommendations, followed by incubation with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich). The sections were then counterstained with hematoxylin. Images were acquired using a Mirax Scan slide scanner (Zeiss) at ×20 magnification. Section images were analyzed by a scientist blinded to the treatment conditions using ImageJ (NIH, Bethesda, Maryland). Sections for which all

three valves were present were quantified and averaged for each mouse. Lesion area was manually traced for each section, quantified in μm^2 and reported as a percentage of the total vessel area to account for differences in total aortic sinus cross-sectional area between mice. The endothelial surface was manually traced with a 20 μm thick line and VCAM1-positive staining was reported as a percentage of this line area. CD45-positive staining was quantified only in the lesions and reported as a percentage of total lesion area.

2.13 | Proteomic analysis

EOC cell pellets were lysed with RIPA buffer and 100 μg of protein was digested using the FASP method[44] on 30 kDa spin filters (Millipore). The eluted peptides were acidified and desalted using in-house made C18 pipette tips (10 μg capacity). Analysis was performed on an Easy nLC-1200 coupled to a ThermoQExactive HF mass spectrometer (Thermo Fisher Scientific) operating in a top 20 mode. The mobile phase was composed of Buffer A (0.1% formic acid) and Buffer B (0.1% formic acid in 80% acetonitrile). Peptides were separated using a PepMap RSLC C18 2 μm, 75 μm × 50 cm column and a PepMap 100 C18 3 μm, 75 μm × 2 cm precolumn with a 2 hour gradient of 5% to 40% Buffer B. Data were analyzed using MaxQuant (v1.6.10.43)[45] and Perseus.[46]

2.14 | Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 6; GraphPad Software Inc., La Jolla, California). Data are presented as mean ± SD or SEM, as indicated in the figure legends. Data with n ≥8 were subjected to the D'Agostino and Pearson omnibus normality test and found to conform to a normal distribution. One-way ANOVA, followed by the Holm's Sidak's post hoc test, was used for comparison between more than two groups. For the human EOC study, no sample size calculation was performed as the analysis was considered exploratory. A parametric paired two-tailed *t* test was used to compare treatment groups in the human EOC studies. A value of $P < .05$ was considered to be statistically significant.

3 | RESULTS

3.1 | GW3965 alters differentiation of EOCs to the endothelial lineage

We previously demonstrated using a loss-of-function model that LXRs play an important role in mitigating the negative effects of cholesterol, either from Western diet feeding or addition of exogenous cholesterol, on EOC differentiation.[41] Here, we set out to determine whether LXR activation would have an opposing role on EOC differentiation. Treatment with LXR agonist (1 μM GW3965) throughout EOC differentiation decreased the expression of the endothelial lineage markers *Cd144* and *Vegfr2* both by 76% in day 7 EOCs derived from WT mice, but not *Lxrαβ−/−* mice ($P < .001$; Figure 1A,B). Interestingly, GW3965 treatment resulted in a varied time course profile for these endothelial genes. WT cells differentiated in the presence of GW3965 showed similar expression of *Cd144* compared to vehicle treated cells up to day 3. However, after day 3, *Cd144* expression was decreased in the WT GW3965-treated EOCs compared to vehicle treatment, suggesting a de-differentiation program (Figure 1A). On the other hand, the increase in *Vegfr2* expression normally observed with differentiation was prevented in WT cells treated with GW3965 (Figure 1B).

We next examined the expression of leukocyte and myeloid markers, which are expected to decrease during differentiation. Time course analysis did reveal potent reductions of the pan leukocyte marker *Cd45* by day 3 of differentiation, and while GW3965 treatment appeared to delay this decrease at day 7 in EOCs, this effect was not LXR-dependent (Figure 1C). Furthermore, at day 7, expression of the myeloid marker *Cd11b* was not altered by GW3965 in WT EOCs (Supporting Information Figure S1A). During atherosclerosis, endothelial to mesenchymal transition (EndMT) induced by oxidative stress, TGFβ, and other stressors characteristic of the atherosclerotic milieu, can cause endothelial cells to acquire a more fibrotic phenotype and express mesenchymal markers such as *Tgfβ* and *Fsp-1*.[47,48] Notably, while LXR activation decreased EOC expression of endothelial lineage markers (*Cd144* and *Vegfr2*), expression of these mesenchymal markers in the GW3965-treated WT EOCs was not increased (Supporting Information Figure S1B,C), suggesting that these EOCs were not undergoing EndMT. These data indicate that while GW3965 treatment to WT cells decreased differentiation to the endothelial lineage, expression of the leukocyte/myeloid markers *Cd45* and *Cd11b* were not increased, suggesting that agonist treatment did not prevent differentiation or induce de-differentiation, but rather produced an altered population of EOCs.

To assess the effect of GW3965 at the protein level we performed shotgun proteomics on the EOCs after 1 day or 9 days of differentiation. While CD144 and VEGFR2 proteins were not detectable, we did observe increased EOC expression of another classic endothelial marker, von Willebrand factor (vWF) during differentiation (day 1 vs day 9). Similar to the trends observed for other endothelial markers at the gene expression level, EOC differentiation in the presence of GW3965 resulted in decreased levels of vWF compared to vehicle (Supporting Information Figure S1D). Unsupervised clustering of the data demonstrated that each of the three treatment groups clustered separately, supporting the idea that the GW3965 treatment creates a distinct EOC sub-population (Supporting Information Figure S1E).

3.2 | Secretome of GW3965-treated EOCs reduced monocyte endothelial adhesion

To investigate the impact of LXR-activated EOCs during the early stages of atherosclerosis, we used the in vitro monocyte-endothelium

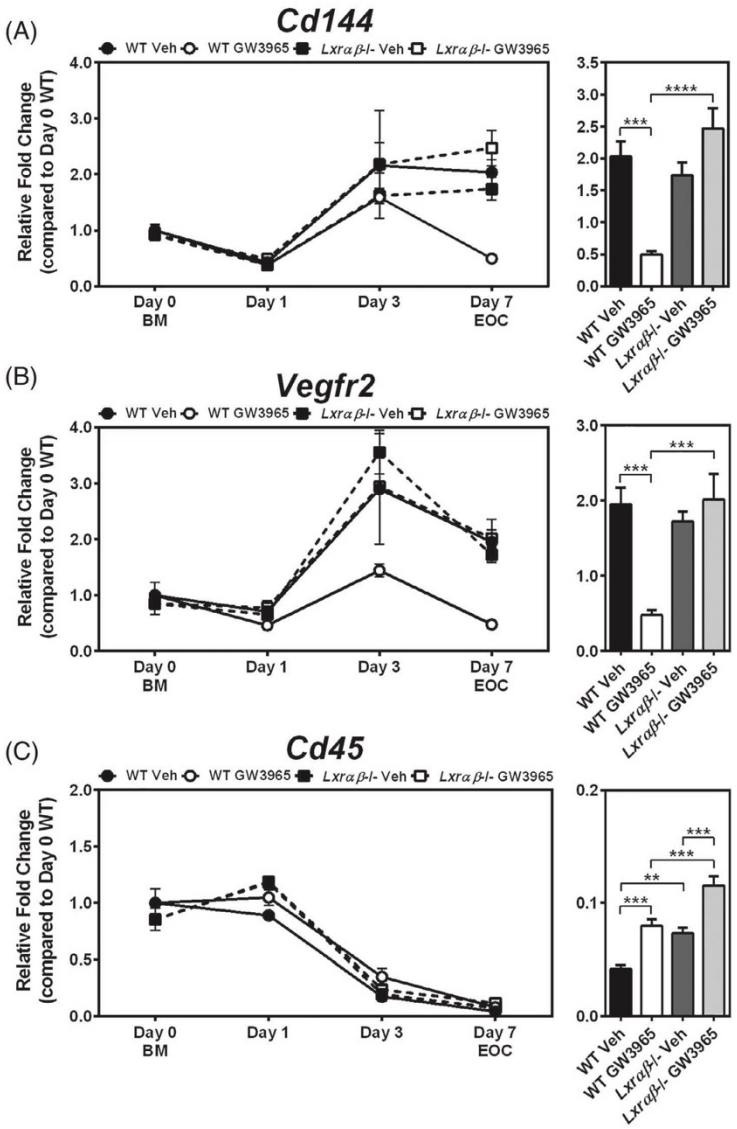


FIGURE 1 Wild-type EOCs treated with LXR agonist have lower expression of endothelial markers after differentiation. A-C, Bone marrow (BM; Day 0) from 12-week old WT and *Lxrαβ* −/− mice were differentiated for 7 days in the presence of LXR agonist (1 μM GW3965). Gene expression was performed for the endothelial lineage markers (A) *Cd144* and (B) *Vegfr2*, as well as (C) the leukocyte marker *Cd45*. Time course analyses are shown in the left panels, and day 7 EOC expression levels in the right panel. n = 5-6 per group. Data represent the mean ± SEM. $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$

adhesion assay. HUVECs activated with TNFα were incubated with CM from vehicle- or GW3965-treated EOCs prior to the addition of fluorescently labeled THP-1 monocytes. As expected, TNFα-treatment increased monocyte adhesion to HUVECs by 8.8-fold ($P < .0001$; Figure 2A). Treatment of HUVECs with CM from GW3965-treated WT EOCs (WT GW CM) decreased the adhesion of THP-1 cells by 36% compared to WT Veh CM ($P < .0001$; Figure 2A). In contrast, CM from GW3965-treated *Lxrαβ*−/− EOCs (*Lxr*−/− GW CM) had no effect on TNFα-induced monocyte adherence. These results indicate that LXR activation altered the EOC population, which in turn produced a secretome that decreased monocyte adhesion to activated endothelial cells.

During atherosclerosis, endothelial cells upregulate the expression of selectins, which facilitate leukocyte rolling, and adhesion molecules that enhance leukocyte adhesion to the aortic endothelium.[3] To determine whether WT GW CM decreased monocyte adhesion by altering the expression of adhesion molecules via signaling to the HUVECs, we performed gene expression analysis of the HUVECs after incubation with

EOC CM. We observed that while the addition of the pro-inflammatory stimulus TNFα did induce the expression of the adhesion molecules *VCAM1* and *ICAM1*, as well as *SELE*, there was no change in any of these markers of endothelial activation when co-treated with WT GW CM compared the WT Veh CM (Supporting Information Figure S2A). To establish whether the CM was required to "prime" the endothelial cells prior to TNFα administration, we tested whether CM administered only in the pre-TNFα period (20 hours) and then washed out; or only during TNFα treatment (ie, for 4 hours concurrent with TNFα) would be sufficient to elicit the beneficial effect to decrease monocyte-endothelial binding. Interestingly, CM was effective at decreasing monocyte adhesion under both conditions and the effect was most pronounced when included both before and during TNFα (Supporting Information Figure S2D). These data suggest that components in the secretome can both neutralize the effect of TNFα and prime the endothelial cells to protect them from TNFα-mediated activation. The detailed mechanisms by which WT GW CM alters monocyte-HUVEC adhesion, however, remain elusive.

We next set out to determine whether the therapeutic effects of the GW3965 on the EOC secretome could be translated to patients with CAD. For this, we obtained peripheral blood samples from a CAD cohort with characteristics described in Table 1. Cells were differentiated to EOCs in the presence of vehicle or 1 μM GW3965 and the CM was collected at day 7. Similar to EOCs derived from mouse bone marrow, monocyte adhesion was decreased by 54% when HUVECs were treated with the CM from GW3965-treated EOCs from the CAD patients compared to vehicle treatment ($P < .0001$; Figure 2B). This therefore demonstrated that GW3965 improved EOC function from CAD patients to a similar extent as that observed in murine cells from WT mice.

3.3 | Administration of EOCs and their secreted factors reduce plaque size during atherogenesis

Ex vivo cultured EOCs are currently undergoing clinical trials for the treatment of vascular complications arising from cardiovascular disease, such as pulmonary hypertension and acute myocardial infarction.[38-40] In our study, we wanted to assess the therapeutic potential of WT GW3965-treated EOCs and their secretome on the development of atherosclerosis using the atherogenic *Ldlr*-knockout (*Ldlr*−/−) mouse model (Figure 3A). The *Ldlr*−/− mice receiving EOCs were splenectomized prior to initiation of the treatments to prevent sequestration of EOCs within the spleen.[49] Plasma cholesterol levels were not changed with EOC administration (Figure 3B). Likewise, the number of circulating leukocytes were unchanged in *Ldlr*−/− mice with EOC administration compared to saline injected mice (Supporting Information Table S3). Histological analysis of the aortic sinus revealed a reduction in the luminal plaque coverage of the *Ldlr*−/− mice receiving the GW3965-treated EOCs compared to those receiving the Veh-treated EOCs (44%, $P < .05$) or saline (41%, n.s.) (Figure 3C). Despite these reductions in plaque area, there were no changes in endothelial activation (VCAM1) or leukocytes (CD45) within the plaque upon the administration of GW3965-treated EOCs (Figure 3C).

Since our in vitro results found a potent effect of the EOC secretome on limiting monocyte-endothelial adhesion (Figure 2A), we administered CM from treated EOCs or control (unconditioned) media bi-weekly to *Ldlr*−/− mice (with an intact spleen) to determine whether this alone would be sufficient to impact lesion formation (Figure 4A). We did not observe changes in plasma cholesterol upon injection of the CM (Figure 4B). Likewise, there was no effect on total blood counts of mice treated with CM, with the exception of mice

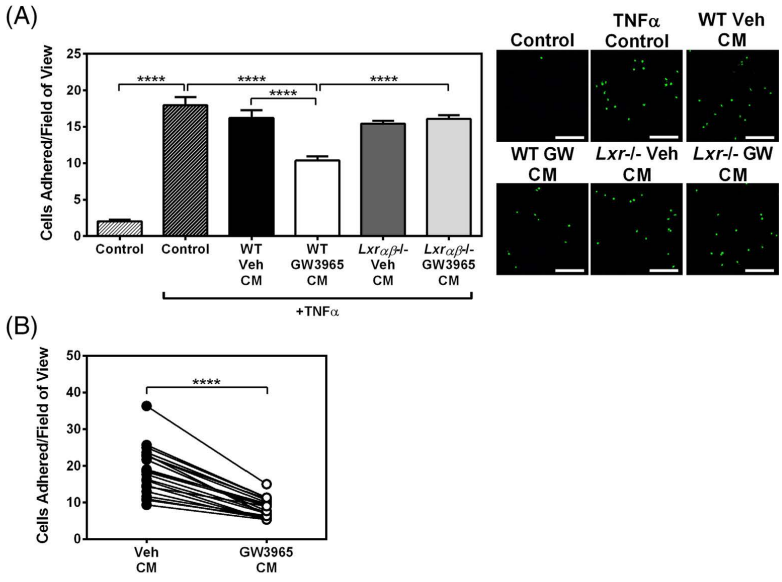


FIGURE 2 Secreted factors from GW3965-treated mouse and patient-derived EOCs reduce monocyte adherence to activated endothelial cells. A,B, Conditioned media from GW3965-treated EOCs were harvested and applied to HUVECs treated with TNFα. Monocyte adhesion to treated HUVECs was quantified after incubation with conditioned media derived from (A) WT and *Lxrαβ*−/− mouse EOCs (n = 8 per group) and (B) EOCs derived from patients with coronary artery disease (n = 21 patients). Scale bar = 200 μm. Data represent the mean ± SEM. ****$P < .0001$

receiving CM from GW3965-treated EOCs in which monocytes were unexpectedly increased (Supporting Information Table S4). Remarkably, treatment with CM from GW3965-treated EOCs reduced atherosclerotic plaques in the aortic sinus by 36% ($P < .01$) compared to Veh-treated EOC CM and 47% ($P < .001$) compared to control unconditioned EBM-2 media (Figure 4C). The CM from the GW3965-treated EOCs significantly reduced VCAM1, the adhesion molecule responsible for leukocyte adhesion to the inflammatory endothelium during atherogenesis (Figure 4C). The proportions of CD45, a pan-leukocyte marker, were not altered; indicating in part that the CM did not alter the content of the plaque but rather through the effects on the endothelium reduced total lesion area. No differences in plasma IL-6 levels or spleen weights were found among the treatment groups (Supporting Information Figure S3),

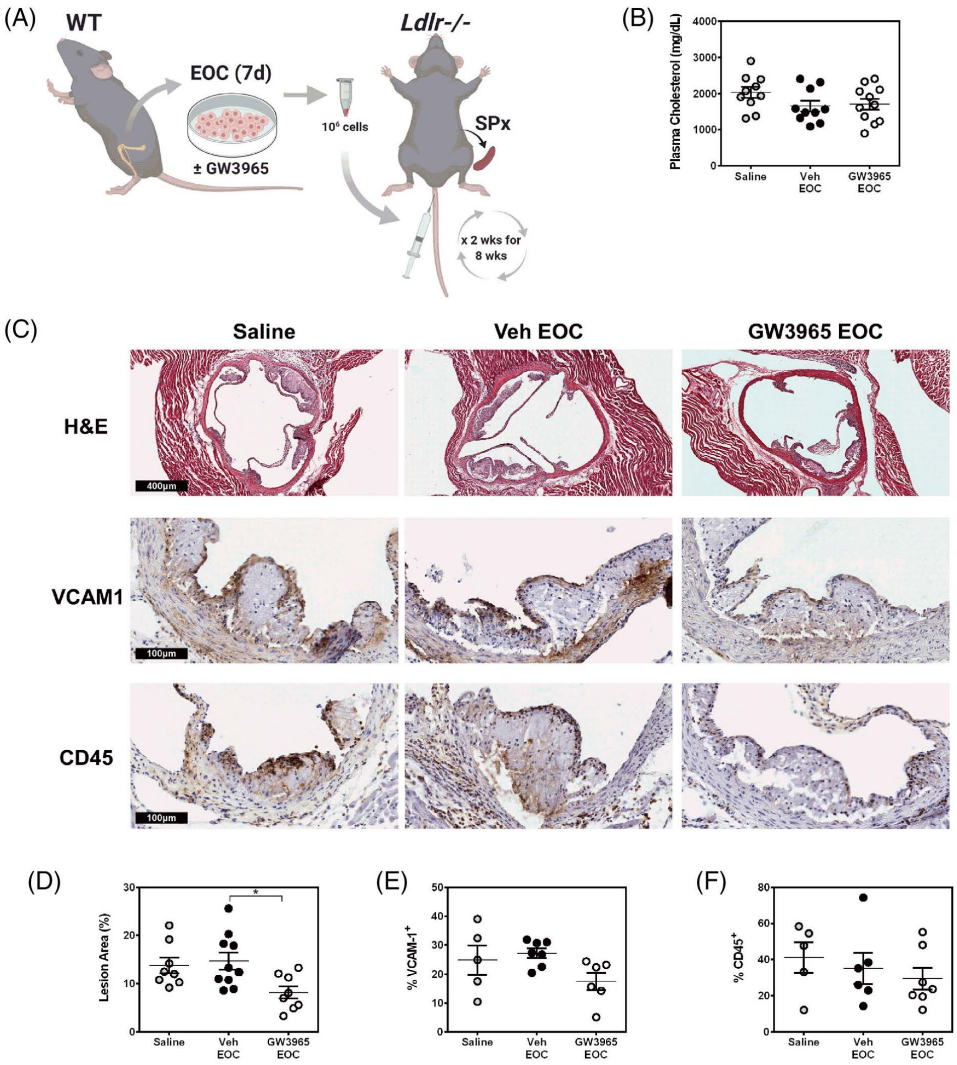


FIGURE 3 LXR agonist-treated EOCs reduce plaque area in the aortic sinus. A, EOCs derived from WT mice were differentiated in the presence of GW3965 and administered to splenectomized *Ldlr−/−* mice bi-weekly over an 8-week period. B, Plasma cholesterol levels were quantified from the blood collected at sacrifice. C-F, Histological analyses were performed on the aortic sinus sections for total lesion area (C, D) and percent positive areas for VCAM1 (C, E) and CD45 (C, F). n = 5-11 mice per group. Data represent the mean ± SEM. *$P < .05$

suggesting no overall effect on systemic inflammatory status. While the molecular component(s) contributing to these changes are not clear, these data demonstrate the therapeutic potential of the GW3965-treated EOC secretome to limit the development of atherosclerosis in mice, with in vitro evidence that this response will be conserved in humans.

4 | DISCUSSION

Atherosclerosis is a progressive disease that manifests over several decades. The gold standard treatment for atherosclerosis relies on lipid-lowering, such as the administration of statin drugs and more recently, PCSK9 inhibitors.[50] However, results from the Canakinumab

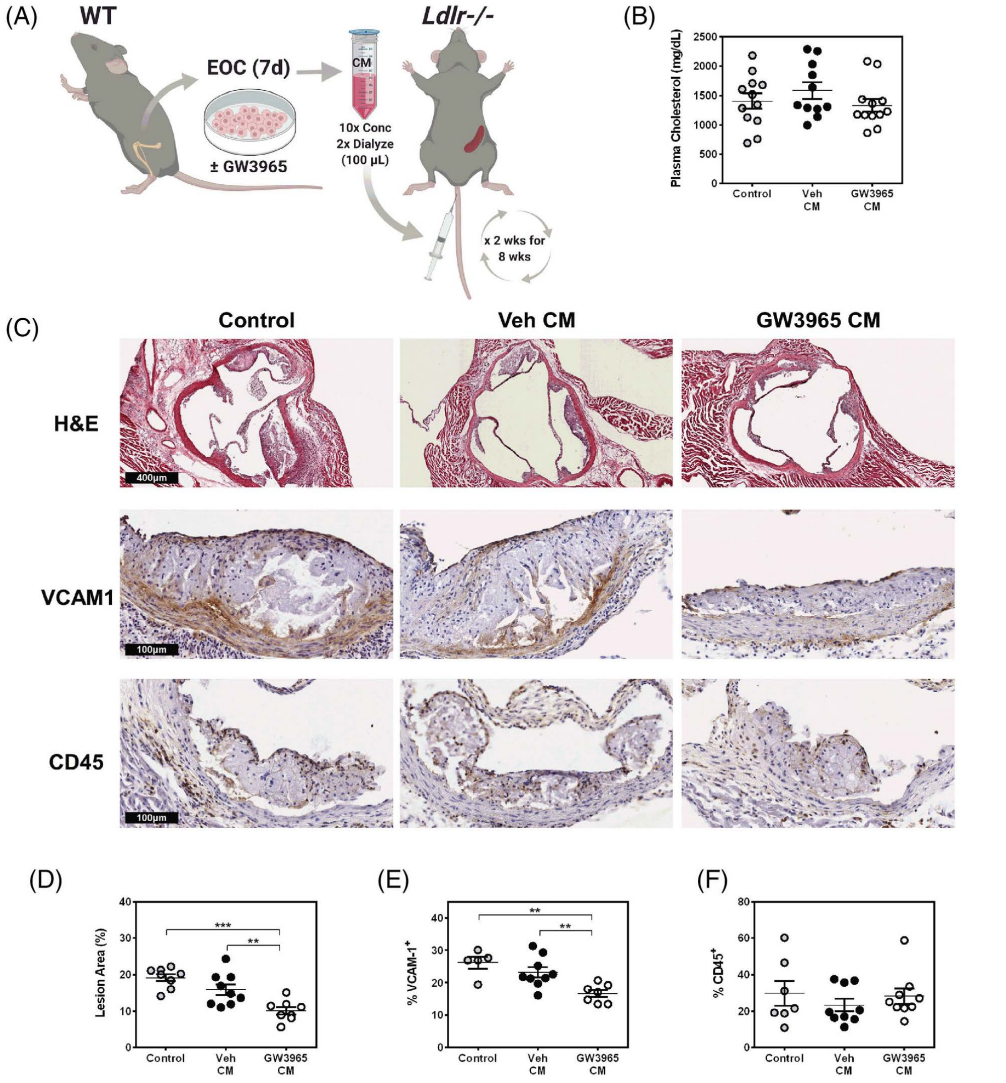


FIGURE 4 The secretome of GW3965-treated EOCs decreases atherosclerotic plaque burden. A, The conditioned media from GW3965 treated EOCs were harvested and administered to *Ldlr*−/− mice bi-weekly over 8 weeks. B, Plasma cholesterol levels were assessed in samples collected at sacrifice. C-F, Histological analyses were performed on the aortic sinus sections for total lesion area (C, D) and percent positive areas for VCAM1 (C, E) and CD45 (C, F). n = 5-12 mice per group. Data represent the mean ± SEM. $^{**}P < .01$, $^{***}P < .001$

Anti-inflammatory Thrombosis Outcome Study (CANTOS) trial found that targeting the inflammatory axis can reduce cardiovascular-related mortality, independent of lipid lowering.[50,51] These data, among others, emphasize the opportunity for the development of novel therapeutic avenues for the treatment of atherosclerosis that go beyond traditional lipid lowering modalities.

During the early stages of atherosclerosis, damage that occurs within the atherosclerotic environment overwhelms endogenous endothelial repair mechanisms, resulting in an overall pro-inflammatory response that allows for an increase in endothelial expression of adhesion molecules that facilitate leukocyte binding.[3] While the endothelium has the capability to repair itself, endothelial repair can also be supported by EOCs through the secretion of vasoreparative factors. We previously described a role for LXRs in mitigating the negative effects of high cholesterol-induced defects on EOC differentiation and the secretome.[41] Given the importance of EOCs in mediating endothelial repair via the secretome and the contributions of endothelial activation to the early pathogenesis of atherosclerosis, we tested whether pharmacologic activation of LXRs in EOCs produces a secretome that could reduce the progression of early stage atherosclerosis. Our data here show that LXR activation during EOC differentiation decreases the expression of endothelial lineage markers. This shift in differentiation qualitatively alters the secretome of the GW3965-treated EOCs in a favorable manner, resulting in less monocyte-endothelial adherence and an overall decrease in atherosclerosis. Along with our previously published data,[41] this therefore provides a model whereby expression of endothelial markers is inversely associated with the vasoreparative benefit of the EOC secretome.

In contrast to the loss-of-function model, in which increased cholesterol in the *Lxrαβ−/−* EOCs correlated with increased expression of endothelial lineage markers[41]; the gain-of-function LXR model studied here demonstrated a decrease in these endothelial markers in the absence of changes in EOC cholesterol content (data not shown). These data suggest that there are separate mechanisms regulating EOC differentiation under the influence of cholesterol when LXRs are absent compared to when they are activated. Pharmacologic activation of LXRs throughout differentiation specifically decreased the expression of the endothelial lineage markers *Cd144* and *Vegfr2*. Time course analysis revealed that GW3965 treatment prevented the increase of *Vegfr2*, while *Cd144* expression decreased after day 3 of differentiation, compared to vehicle-treated cells. Further experiments will be needed to confirm the mechanism by which EOC differentiation to the endothelial lineage is impacted by the activation of LXR, and how this occurs for each of these endothelial markers. These experiments will need to determine whether LXRs are directly binding to the DNA to inhibit endothelial marker expression or interact with other factors to inhibit transcriptional machinery at these individual loci.

We attempted to narrow down potential mechanisms by which GW3965-treated EOC derived CM decreased monocyte to endothelial adhesion using gene expression analysis of HUVECs. We found no significant differences in marker genes associated with enhanced adhesion. While VCAM1 was not downregulated by treatment with the CM from GW3965-treated EOCs in culture, administration of the CM to atherosclerosis-prone mice did show a downregulation of endothelial VCAM1 in the aortic sinus, which was associated with a decrease in plaque area. The discrepancy between our in vitro and in vivo results could be due to exposure time (ie, in vitro the HUVEC cells were exposed to CM for only 24 hours vs in vivo CM was injected every 2 weeks for 8 weeks) or due to indirect effects of the GW3965 CM on cell types apart from endothelial cells. Nevertheless, the precise mechanism by which the secretome exerts this effect on the endothelium to mitigate lesion development is yet to be identified. Signals transduced from the secretome are likely derived from proteins[24,52-57] or components of extracellular vesicles (ie, miRNAs).[58-60] As such, investigation and fractionation of the LXR-dependent changes to the EOC secretome is warranted to identify potential effector pathways of endothelial cells that lead to decreased monocyte adhesion, including unbiased proteomic approaches and analysis of RNA species contained within the ligand-treated EOC secretome. A preliminary proteomic analysis of unfractionated day 7 EOC secretomes found elevated levels of several known LXR targets in the CM from GW3965-treated vs vehicle-treated cells. For example, phospholipid transfer protein (PLTP), apoptotic inhibitory factor AIM/CD5L, IL18 binding protein (IL18BP) and lipoprotein lipase (LPL) were increased over 2-fold. Intriguingly, among these secreted proteins, PLTP, CD5L, and LPL have been reported to exacerbate or be associated with advanced atherosclerosis,[61-64] whereas IL18BP is anti-atherogenic.[65] Further experiments are needed to fractionate and assess all candidates alone or in combination to determine how, if any, exert their protective effect in the CM. Nevertheless, we show here that GW3965-treatment of EOCs derived from patients with CAD produces a secretome that also reduces monocyte-endothelial adhesion (Figure 2B), indicating that LXR agonist treatment to EOCs could potentially be a translatable therapeutic for patients with cardiovascular disease.

The administration of EOCs to atherosclerotic mouse models has been previously shown to promote plaque stabilization and decrease plaque formation, primarily through endothelial engraftment.[66-69] However, those studies were all performed in mouse models of plaque regression that targeted the later stages of atherosclerosis. EOC intervention at the early stages of atherosclerosis, as described herein, has not, to our knowledge, been previously explored. As a complement to our in vitro monocyte-endothelial adhesion assay, we evaluated the therapeutic potential of LXR treatment to EOCs in a mouse model of early atherosclerosis. In line with numerous studies and clinical trials (reviewed by Mindur and Swirski[70]) that show the potency of circulating growth factors and cytokines in promoting and/or limiting the development of atherosclerosis, administration of the secreted factors from GW3965-treated EOCs significantly reduced aortic lesion development in recipient *Ldlr−/−* mice. Of note, the effect of injecting GW3965-treated EOCs vs GW3965-treated CM on lesion formation was very similar (44% vs 36%, respectively; Figures 3C and 4C) indicating that the majority of the beneficial effect

is derived from the secretome. These data are in line with recent reports in which the EOC secretome has been packaged in nanoparticles or hydrogels to treat acute ischemic diseases.[71,72]

Previous studies determined that in the absence of the cholesterol efflux transporters (*Abca1* and *Abcg1*) in myeloid cells of the bone marrow was insufficient to explain the anti-atherogenic effects of LXRs described in whole bone marrow transplant experiments.[9-14] Our data support the role of another effector cell (EPC) in contributing to the anti-atherogenic effects of LXR. Thus, these studies enhance our understanding of LXR cell targets in the bone marrow and provide a unique mechanism to inhibit the development of atherosclerosis. A major drawback to the in vivo use of LXR agonists is the development of hypertriglyceridemia and hepatosteatosis via upregulation of the LXR target gene and lipogenic factor *Srebp1c*, in the liver.[73-75] Our data demonstrate that the cell-free secretome of LXR-treated EOCs is sufficient to protect against endothelial damage and early atherosclerosis. These data therefore support a role for ex vivo treatment of patient-derived EOCs as an avenue of intervention that would elicit the anti-atherogenic effects of LXRs in the EOCs without the hepatosteatosis associated with systemic LXR agonism.

5 | CONCLUSION

Our data demonstrate the potential of ex vivo treatment of patient-derived EOCs with LXR agonists as a novel therapy to decrease the endothelial defects associated with atherosclerosis and reduce plaque burden during its progression. Further experiments will need to be performed to examine the long-term efficacy and any potential toxicity that may be associated with administering CM prior to human studies.

ACKNOWLEDGMENTS

We would like to sincerely thank Dr. David J. Mangelsdorf (University of Texas Southwestern Medical Center, Dallas, Texas) for kindly providing the liver X receptor knockout mice, Dr. Erin E. Mulvihill for her guidance with the animal sacrifices, and Xiaoling Zhao for processing samples for histology. This work was supported by grants-in-aid from the Heart and Stroke Foundation of Canada (Carolyn L. Cummins, G-13-0002612 and G-18-0022364), the Ontario Graduate Scholarship (Adil Rasheed and Michael F. Saikali), and the Dean's Fund of the Leslie Dan Faculty of Pharmacy (Sarah A. Shawky).

CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

A.R.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; S.A.S.: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript; R.T., M.F.S.: collection and/or assembly of data, final approval of manuscript; R.G.J., T.S., B.H.: provision of study material or patients, collection and/or assembly of data, final approval of manuscript; K.J.R.: data analysis and interpretation, final approval of manuscript; C.L.C.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository[76] with the dataset identifier PXD021993.

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REFERENCES

1. Writing Group Members, Mozaffarian D, Benjamin EJ, et al. Heart disease and stroke statistics-2016 update: a report from the American Heart Association. *Circulation*. 2016;133(4):e38-e360.
2. Wang JC, Bennett M. Aging and atherosclerosis: mechanisms, functional consequences, and potential therapeutics for cellular senescence. *Circ Res*. 2012;111(2):245-259.
3. Mestas J, Ley K. Monocyte-endothelial cell interactions in the development of atherosclerosis. *Trends Cardiovasc Med*. 2008;18(6):228-232.
4. Song C, Hiipakka RA, Kokontis JM, et al. Ubiquitous receptor: structures, immunocytochemical localization, and modulation of gene activation by receptors for retinoic acids and thyroid hormones. *Ann N Y Acad Sci*. 1995;761:38-49.
5. Willy PJ, Umesono K, Ong ES, Evans RM, Heyman RA, Mangelsdorf DJ. LXR, a nuclear receptor that defines a distinct retinoid response pathway. *Genes Dev*. 1995;9(9):1033-1045.
6. Repa JJ, Turley SD, Lobaccaro JA, et al. Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. *Science*. 2000;289(5484):1524-1529.
7. Klucken J, Buchler C, Orso E, et al. ABCG1 (ABC8), the human homolog of the drosophila white gene, is a regulator of macrophage cholesterol and phospholipid transport. *Proc Natl Acad Sci USA*. 2000;97(2):817-822.
8. Joseph SB, Castrillo A, Laffitte BA, Mangelsdorf DJ, Tontonoz P. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.] [in Eng]. *Nat Med*. 2003;9(2):213-219.
9. Tangirala RK, Bischoff ED, Joseph SB, et al. Identification of macrophage liver X receptors as inhibitors of atherosclerosis [Research Support, Non-U.S. Gov't] [in Eng]. *Proc Natl Acad Sci USA*. 2002;99(18):11896-11901.
10. Levin N, Bischoff ED, Daige CL, et al. Macrophage liver X receptor is required for antiatherogenic activity of LXR agonists. *Arterioscler Thromb Vasc Biol*. 2005;25(1):135-142.
11. van der Stoep M, Li Z, Calpe-Berdiel L, et al. Elimination of macrophages drives LXR-induced regression both in initial and advanced stages of atherosclerotic lesion development. *Biochem Pharmacol*. 2013;86(11):1594-1602.
12. Feig JE, Pineda-Torra I, Sanson M, et al. LXR promotes the maximal egress of monocyte-derived cells from mouse aortic plaques during atherosclerosis regression. *J Clin Invest*. 2010;120(12):4415-4424.
13. Bischoff ED, Daige CL, Petrowski M, et al. Non-redundant roles for LXRalpha and LXRbeta in atherosclerosis susceptibility in low density lipoprotein receptor knockout mice. *J Lipid Res*. 2010;51(5):900-906.

14. Rasheed A, Cummins CL. Beyond the foam cell: the role of LXRs in preventing atherogenesis. *Int J Mol Sci*. 2018;19(8).1–20.
15. Westerterp M, Murphy AJ, Wang M, et al. Deficiency of ATP-binding cassette transporters A1 and G1 in macrophages increases inflammation and accelerates atherosclerosis in mice. *Circ Res*. 2013;112(11): 1456-1465.
16. Kappus MS, Murphy AJ, Abramowicz S, et al. Activation of liver X receptor decreases atherosclerosis in Ldlr(−)/(−) mice in the absence of ATP-binding cassette transporters A1 and G1 in myeloid cells. *Arterioscler Thromb Vasc Biol*. 2014;34(2):279-284.
17. Bi X, Zhu X, Gao C, et al. Myeloid cell-specific ATP-binding cassette transporter A1 deletion has minimal impact on atherogenesis in atherogenic diet-fed low-density lipoprotein receptor knockout mice. *Arterioscler Thromb Vasc Biol*. 2014;34(9):1888-1899.
18. Seita J, Weissman IL. Hematopoietic stem cell: self-renewal versus differentiation. *Wiley Interdiscip Rev Syst Biol Med*. 2010;2(6):640-653.
19. Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science*. 1997;275(5302):964-967.
20. Yang J, Ii M, Kamei N, et al. CD34+ cells represent highly functional endothelial progenitor cells in murine bone marrow. *PLoS One*. 2011; 6(5):e20219.
21. Fadini GP, Miorin M, Facco M, et al. Circulating endothelial progenitor cells are reduced in peripheral vascular complications of type 2 diabetes mellitus. *J Am Coll Cardiol*. 2005;45(9):1449-1457.
22. Vasa M, Fichtlscherer S, Aicher A, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res*. 2001;89(1):E1-E7.
23. Schmidt-Lucke C, Rossig L, Fichtlscherer S, et al. Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events: proof of concept for the clinical importance of endogenous vascular repair. *Circulation*. 2005;111(22):2981-2987.
24. Hur J, Yoon CH, Kim HS, et al. Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. *Arterioscler Thromb Vasc Biol*. 2004;24(2): 288-293.
25. Cheng CC, Chang SJ, Chueh YN, et al. Distinct angiogenesis roles and surface markers of early and late endothelial progenitor cells revealed by functional group analyses. *BMC Genomics*. 2013;14:182.
26. Urbich C, Dimmeler S. Endothelial progenitor cells: characterization and role in vascular biology. *Circ Res*. 2004;95(4):343-353.
27. Mukai N, Akahori T, Komaki M, et al. A comparison of the tube forming potentials of early and late endothelial progenitor cells. *Exp Cell Res*. 2008;314(3):430-440.
28. Kawamoto A, Gwon HC, Iwaguro H, et al. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. *Circulation*. 2001;103(5):634-637.
29. Kocher AA, Schuster MD, Szabolcs MJ, et al. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med*. 2001;7(4):430-436.
30. Orlic D, Kajstura J, Chimenti S, et al. Transplanted adult bone marrow cells repair myocardial infarcts in mice. *Ann N Y Acad Sci*. 2001;938: 221-229. discussion 229-230.
31. Orlic D, Kajstura J, Chimenti S, et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. *Proc Natl Acad Sci USA*. 2001;98(18):10344-10349.
32. Grant MB, May WS, Caballero S, et al. Adult hematopoietic stem cells provide functional hemangioblast activity during retinal neovascularization. *Nat Med*. 2002;8(6):607-612.
33. Zhang ZG, Zhang L, Jiang Q, Chopp M. Bone marrow-derived endothelial progenitor cells participate in cerebral neovascularization after focal cerebral ischemia in the adult mouse. *Circ Res*. 2002;90(3):284-288.
34. Hagensen MK, Shim J, Thim T, Falk E, Bentzon JF. Circulating endothelial progenitor cells do not contribute to plaque endothelium in murine atherosclerosis. *Circulation*. 2010;121(7):898-905.
35. Crosby JR, Kaminski WE, Schatteman G, et al. Endothelial cells of hematopoietic origin make a significant contribution to adult blood vessel formation. *Circ Res*. 2000;87(9):728-730.
36. Ziegelhoeffer T, Fernandez B, Kostin S, et al. Bone marrow-derived cells do not incorporate into the adult growing vasculature. *Circ Res*. 2004;94(2):230-238.
37. Shantsila E, Watson T, Lip GY. Endothelial progenitor cells in cardiovascular disorders. *J Am Coll Cardiol*. 2007;49(7):741-752.
38. Granton J, Langleben D, Kutryk MB, et al. Endothelial NO-synthase gene-enhanced progenitor cell therapy for pulmonary arterial hypertension: the PHACeT trial. *Circ Res*. 2015;117(7):645-654.
39. Taljaard M, Ward MR, Kutryk MJ, et al. Rationale and design of Enhanced Angiogenic Cell Therapy in Acute Myocardial Infarction (ENACT-AMI): the first randomized placebo-controlled trial of enhanced progenitor cell therapy for acute myocardial infarction. *Am Heart J*. 2010;159(3):354-360.
40. Chong MS, Ng WK, Chan JK. Concise review: endothelial progenitor cells in regenerative medicine: applications and challenges. *Stem Cells Translational Medicine*. 2016;5(4):530-538.
41. Rasheed A, Tsai R, Cummins CL. Loss of the liver X receptors disrupts the balance of hematopoietic populations, with detrimental effects on endothelial progenitor cells. *J Am Heart Assoc*. 2018;7(10):e007787.
42. Jung RG, Simard T, Di Santo P, et al. Performance of plasminogen activator inhibitor-1 as a biomarker in patients undergoing coronary angiography: analytical and biological considerations. *Diab Vasc Dis Res*. 2019;16(5):478–482.
43. Simard T, Jung R, Labinaz A, et al. Evaluation of plasma adenosine as a marker of cardiovascular risk: analytical and biological considerations. *J Am Heart Assoc*. 2019;8(15):e012228.
44. Wisniewski JR, Rakus D. Multi-enzyme digestion FASP and the 'Total protein approach'-based absolute quantification of the *Escherichia coli* proteome [Research Support, Non-U.S. Gov't] [in Eng]. *J Proteomics*. 2014;109:322-331.
45. Tyanova S, Temu T, Cox J. The MaxQuant computational platform for mass spectrometry-based shotgun proteomics [in Eng]. *Nat Prot*. 2016;11(12):2301-2319.
46. Tyanova S, Temu T, Sinitcyn P, et al. The Perseus computational platform for comprehensive analysis of (prote)omics data [Research Support, Non-U.S. Gov't] [in Eng]. *Nat Methods*. 2016;13(9):731-740.
47. Evrard SM, Lecce L, Michelis KC, et al. Endothelial to mesenchymal transition is common in atherosclerotic lesions and is associated with plaque instability. *Nat Commun*. 2016;7:11853.
48. Kovacic JC, Mercader N, Torres M, Boehm M, Fuster V. Epithelial-to-mesenchymal and endothelial-to-mesenchymal transition: from cardiovascular development to disease. *Circulation*. 2012;125(14):1795-1808.
49. Zhao X, Qian D, Wu N, et al. The spleen recruits endothelial progenitor cell via SDF-1/CXCR4 axis in mice. *J Recept Signal Transduct Res*. 2010;30(4):246-254.
50. Libby P. Interleukin-1 beta as a target for atherosclerosis therapy: biological basis of CANTOS and beyond. *J Am Coll Cardiol*. 2017;70(18): 2278-2289.
51. Ridker PM, Everett BM, Thuren T, et al. Antiinflammatory therapy with canakinumab for atherosclerotic disease. *N Engl J Med*. 2017; 377(12):1119-1131.
52. Rehman J, Li J, Orschell CM, March KL. Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. *Circulation*. 2003;107(8):1164-1169.
53. He T, Smith LA, Harrington S, Nath KA, Caplice NM, Katusic ZS. Transplantation of circulating endothelial progenitor cells restores endothelial function of denuded rabbit carotid arteries. *Stroke*. 2004; 35(10):2378-2384.
54. Urbich C, Aicher A, Heeschen C, et al. Soluble factors released by endothelial progenitor cells promote migration of endothelial cells

and cardiac resident progenitor cells. *J Mol Cell Cardiol*. 2005;39(5):733-742.
55. Pula G, Mayr U, Evans C, et al. Proteomics identifies thymidine phosphorylase as a key regulator of the angiogenic potential of colony-forming units and endothelial progenitor cell cultures. *Circ Res*. 2009;104(1):32-40.
56. Hemmen K, Reinl T, Buttler K, et al. High-resolution mass spectrometric analysis of the secretome from mouse lung endothelial progenitor cells. *Angiogenesis*. 2011;14(2):163-172.
57. Lee MJ, Kim J, Lee KI, Shin JM, Chae JI, Chung HM. Enhancement of wound healing by secretory factors of endothelial precursor cells derived from human embryonic stem cells. *Cytotherapy*. 2011;13(2):165-178.
58. Burger D, Vinas JL, Akbari S, et al. Human endothelial colony-forming cells protect against acute kidney injury: role of exosomes. *Am J Pathol*. 2015;185(8):2309-2323.
59. Deregibus MC, Cantaluppi V, Calogero R, et al. Endothelial progenitor cell derived microvesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA. *Blood*. 2007;110(7):2440-2448.
60. Ranghino A, Cantaluppi V, Grange C, et al. Endothelial progenitor cell-derived microvesicles improve neovascularization in a murine model of hindlimb ischemia. *Int J Immunopathol Pharmacol*. 2012;25(1):75-85.
61. Arai S, Shelton JM, Chen M, et al. A role for the apoptosis inhibitory factor AIM/Spalpha/Api6 in atherosclerosis development [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.] [in Eng]. *Cell Metab*. 2005;1(3):201-213.
62. Amezaga N, Sanjurjo L, Julve J, et al. Human scavenger protein AIM increases foam cell formation and CD36-mediated oxLDL uptake [Research Support, Non-U.S. Gov't] [in Eng]. *J Leukoc Biol*. 2014;95(3):509-520.
63. Krauss RM. Phospholipid transfer protein and atherosclerosis: genetic studies take aim at a moving target [Comment Editorial] [in Eng]. *Circulation*. 2010;122(5):452-454.
64. Babaev VR, Fazio S, Gleaves LA, Carter KJ, Semenkovich CF, Linton MRF. Macrophage lipoprotein lipase promotes foam cell formation and atherosclerosis in vivo [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.] [in Eng]. *J Clin Invest*. 1999;103(12):1697-1705.
65. Mallat Z, Corbaz A, Scoazec A, et al. Interleukin-18/interleukin-18 binding protein signaling modulates atherosclerotic lesion development and stability [in Eng]. *Circ Res*. 2001;89(7):E41-E45.
66. Zhang Z, Dong J, Lobe CG, Gong P, Liu J, Liao L. CCR5 facilitates endothelial progenitor cell recruitment and promotes the stabilization of atherosclerotic plaques in ApoE−/− mice. *Stem Cell Res Ther*. 2015;6:36.
67. Yao L, Heuser-Baker J, Herlea-Pana O, et al. Bone marrow endothelial progenitors augment atherosclerotic plaque regression in a mouse model of plasma lipid lowering. *Stem Cells*. 2012;30(12):2720-2731.
68. Herlea-Pana O, Yao L, Heuser-Baker J, et al. Chemokine receptors CXCR2 and CX3CR1 differentially regulate functional responses of bone-marrow endothelial progenitors during atherosclerotic plaque regression. *Cardiovasc Res*. 2015;106(2):324-337.
69. Tousoulis D, Briasoulis A, Vogiatzi G, et al. Effects of direct infusion of bone marrow-derived progenitor cells and indirect mobilization of hematopoietic progenitor cells on atherosclerotic plaque and inflammatory process in atherosclerosis. *Int J Cardiol*. 2013;168(5):4769-4774.
70. Mindur JE, Swirski FK. Growth factors as immunotherapeutic targets in cardiovascular disease. *Arterioscler Thromb Vasc Biol*. 2019;39(7):1275-1287.
71. Felice F, Piras AM, Rocchiccioli S, et al. Endothelial progenitor cell secretome delivered by novel polymeric nanoparticles in ischemic hindlimb. *Int J Pharm*. 2018;542(1–2):82-89.
72. Maki T, Morancho A, Martinez-San Segundo P, et al. Endothelial progenitor cell secretome and oligovascular repair in a mouse model of prolonged cerebral hypoperfusion. *Stroke*. 2018;49(4):1003-1010.
73. Repa JJ, Liang G, Ou J, et al. Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. *Genes Dev*. 2000;14(22):2819-2830.
74. Schultz JR, Tu H, Luk A, et al. Role of LXRs in control of lipogenesis. *Genes Dev*. 2000;14(22):2831-2838.
75. Grefhorst A, Elzinga BM, Voshol PJ, et al. Stimulation of lipogenesis by pharmacological activation of the liver X receptor leads to production of large, triglyceride-rich very low density lipoprotein particles. *J Biol Chem*. 2002;277(37):34182-34190.
76. Perez-Riverol Y, Csordas A, Bai J, et al. The PRIDE database and related tools and resources in 2019: improving support for quantification data [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't] [in Eng]. *Nucleic Acids Res*. 2019;47(D1):D442-D450.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Rasheed A, Shawky SA, Tsai R, et al. The secretome of liver X receptor agonist-treated early outgrowth cells decreases atherosclerosis in *Ldlr*−/− mice. *STEM CELLS Transl Med*. 2021;10:479–491. https://doi.org/10.1002/sctm.19-0390

Appendix F

Physician prediction of 1-year mortality in the cardiac catheterization laboratory: comparison to a validated risk score

Citation information:

Jung RG*, Sarathy K, Simard T, Parlow S, Di Santo P, Moreland R, Jung Y, Abdel-Razek O, Boland P, Russo JJ, Chong AY, So D, Froeschl M, Dick A, Glover C, Labinaz M, Le May M, Hibbert B. Physician prediction of one-year mortality in the cardiac catheterization laboratory: comparisons to a validated risk score. **(2021)**. *Coronary Artery Disease*.

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Author: Kiran Sarathy, Richard Jung, Trevor Simard, et al

Publication: Coronary Artery Disease

Publisher: Wolters Kluwer Health, Inc.

Date: Oct 13, 2020

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Physician prediction of 1-year mortality in the cardiac catheterization laboratory: comparison to a validated risk score

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Background **Physician perception of procedural risk and clinical outcome can affect revascularization decision making. Public reporting of percutaneous coronary intervention outcomes accentuates the need for accuracy in risk prediction in order to avoid a treatment paradox of undertreating the highest risk patients. Our study compares a validated risk score to physician prediction (PP) of 1-year mortality based on clinical impression at the time of invasive angiography.**

Methods and results **We performed a cohort study between August 2015 and May 2018 to determine the discriminative accuracy of interventional cardiologists on one-year mortality of the treated patient. PP of one-year mortality was compared to the New York State Percutaneous Coronary Intervention Reporting System (NYPCIRS) score in predicting mortality. Three thousand seven hundred ninety-two patients were followed with a median follow-up period of 14.4 months (interquartile range 12.4–18.1 months) and 165 patients (4.4%) died within one-year. PP of mortality was associated with one-year mortality with a hazard ratio of 8.78 (95% confidence interval 5.24–14.71, $P < 0.0001$). Clinical presentation in the form of cardiogenic shock, return of spontaneous circulation, and liver and renal dysfunction were associated with PP. Diagnostic accuracy and specificity were improved in PP compared to NYPCIRS. The combination of PP to NYPCIRS improved the overall c-statistic and diagnostic yield.**

Conclusion **PP appears to be especially specific and accurate for prediction of mortality compared to NYPCIRS though it lacks sensitivity. Furthermore, the combination of PP with NYPCIRS improved the c-statistic and diagnostic yield. Overall, the utility of PP with an objective risk score improves the diagnostic accuracy of mortality prediction.**
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Coronary Artery Disease 2020, XXX:000–000

Keywords: mortality,
percutaneous coronary intervention complications, risk stratification

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Received 26 May 2020 Accepted 29 August 2020

Introduction

Physician perception of procedural risk and clinical outcome can influence willingness to perform interventions and resource allocation in treating higher risk cohorts [1]. Broadly, the two main modalities of risk stratification are by subjective clinician gestalt or by objective risk scoring. Comparative data on clinician perception of patient prognosis to established percutaneous coronary intervention (PCI) risk scores is lacking. Data from other sub-specialities including the intensive care unit have demonstrated that at best, physicians are moderately accurate in predicting long-term mortality while cardiology specific data also suggests an association between physician prediction (PP) and established risk scores is limited [2,3].

Accuracy of risk prediction for PCI outcomes forms a crucial basis for decision making regarding the appropriateness and utility of revascularization and initiation

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DOI: 10.1097/MCA.0000000000000967

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of hemodynamic support devices. In the era of public reporting of PCI outcomes, the interplay of physician perception of patient prognosis with the likelihood of patients to receive PCI remains unclear. Indeed, registry data has previously suggested that the highest risk patients undergo invasive coronary angiography less frequently than intermediate-risk patients [4].

It has been speculated that this apparent 'treatment paradox' may be due to risk aversion. In 1992, a coronary angioplasty reporting system, now called the Percutaneous Coronary Interventions Reporting System (PCIRS) was created in New York State, and annual PCIRS reports have been released since 1997. Public release of risk-adjusted mortality statistics has since become mandatory in several other US states in an attempt to monitor the quality of cardiac care being provided. New York PCIRS risk score (NYPCIRS) is a contemporary tool which was based on a Cox proportional hazards model and utilizes nine factors to predict long-term mortality outcomes at 1, 3 and 5 years [3]. In Canada, the Cardiac Care Quality Indicators Report releases institutional but not individual clinician statistics. This point of difference means that specific concerns about clinicians becoming risk-averse due to inadequate adjustment in the highest risk populations being treated are nullified. Such concerns are validated by the observation of a gradual decrease in the rate of PCI for cardiogenic shock in New York following the introduction of public reporting of mortality outcomes [5], despite mounting evidence for early revascularization in cardiogenic shock [6]. Indeed, surveys at the time supported the idea that clinicians would be more likely to avoid treating the most severely unwell patients due to concern that risk adjustment models were limited in their ability to account for the highest risk populations [1].

Our study aimed to compare a validated risk score to PP in ascertaining one-year mortality as determined by clinical impression in patients undergoing invasive coronary angiography and PCI. Specifically, we sought to evaluate the following objectives: (1) to assess the accuracy of interventional cardiologists in their assessment at the time of treatment decision making in comparison to externally validated risk scores, (2) to assess which clinical factors were associated with perception of risk, and (3) to determine whether the addition of standardized risk scoring with NYPCIRS to PP would improve accuracy in identifying patients at highest risk of 12-month mortality and thereby aid clinical decision making.

Methods

Ethics statement

This study was approved by the Ottawa Health Science Network Research Ethics Board (OHSN-REB Protocol #: 20190224-01H) to evaluate clinical outcomes following revascularization.

Patient population, study design and inclusion criteria

The University of Ottawa Heart Institute is a regional revascularization center serving a population of over 1.2 million. All PCI cases are collected in the CAPITAL (Cardiovascular And Percutaneous ClInical TriALs) revascularization registry which indexes over 1200 clinical data points on patients undergoing coronary angiography and intervention with standardized clinical follow-up one-year post-revascularization [7,8].

Baseline characteristics

In the CAPITAL revascularization registry, coronary artery disease (CAD) was defined as obstructive stenoses ≥50% at the time of angiography. Diabetes mellitus was patient-reported or with documentation of hemoglobin A1c ≥6.5% [7]. Tobacco use was dichotomized into smokers (active smoking at the day of procedure) or non-smokers (not smoking at the day of procedure). Positive family history was defined as CAD in a first-degree relative <55 years of age for males and <65 years for females. Dyslipidemia and hypertension were patient-reported or by previous medical history.

Endpoints and risk score categorization

Our primary endpoint was to determine the discriminative accuracy of PP of interventional cardiologist on one-year prognosis of patients compared to NYPCIRS. The procedural cardiologist was asked 'is this patient likely to survive 12 months?' based on their evaluation of all available clinical parameters prior to intervention yielding dichotomized groups of predicted more likely to survive and predicted less likely to survive.

PP of 12-month mortality was compared to the calculated score in accuracy of predicting patient survival at follow-up with NYPCIRS score 10 or greater considered 'higher risk' vs. 9 or lower considered 'lower risk'. NYPCIRS scores were calculated using prospectively collected clinical parameters entered in the registry [3]. The weighting of points behind NYPCIRS risk scoring is found elsewhere [3].

Statistical analysis

Baseline patient characteristics of the cohort are reported as mean ± SD, median ± interquartile range (IQR) or number and percentage (%) where appropriate. Categorical data were analysed using Fisher's exact test or Chi-square testing with data presented as proportions. Continuous variables were analyzed using Student *t*-test.

All patients included in the analysis had their follow-up completed by as outpatient clinic or chart review within 12-months and included in the analysis. The primary outcome of interest was evaluating mortality following angiography by PP and NYPCIRS score. Receiver operating characteristic (ROC) curves were generated for PP or NYPCIRS and we then evaluated the additive value

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of PP to NYPCIRS stratified by ≥10 points compared by net reclassification index and integrated discrimination improvement.

Univariable logistic regression was performed with all known clinical characteristics with a predetermined *P*-value <0.20 to identify variables of interest which influences PP prior to inclusion into a multivariable logistic regression analysis. Clinical characteristics impacting likelihood to receive PCI and mortality were determined a priori and included into the multivariable logistic regression model.

Kaplan–Meier curves were generated to evaluate time-to-event distribution dichotomized by PP and comparisons were made by log-rank tests. Furthermore, hazard ratio (HR) and corresponding 95% confidence intervals (CIs) of PP were obtained from Cox proportional hazards model in both an unadjusted and adjusted model. A post-hoc landmark analysis was conducted after separating events from 0 to 30 days and from 30 days to one-year was conducted to evaluate PP on mortality.

All statistical analyses were performed using SAS v9.4 (SAS Institute Inc., Cary, North Carolina, USA) and all figures were made using GraphPad Prism v8 (GraphPad Software, La Jolla, California, USA). ROC curve and reclassification was performed using the %ROCPLUS macro [9]. Furthermore, piecewise hazards model was analyzed using the %NEWSURV macro [10]. $P < 0.05$ was considered statistically significant.

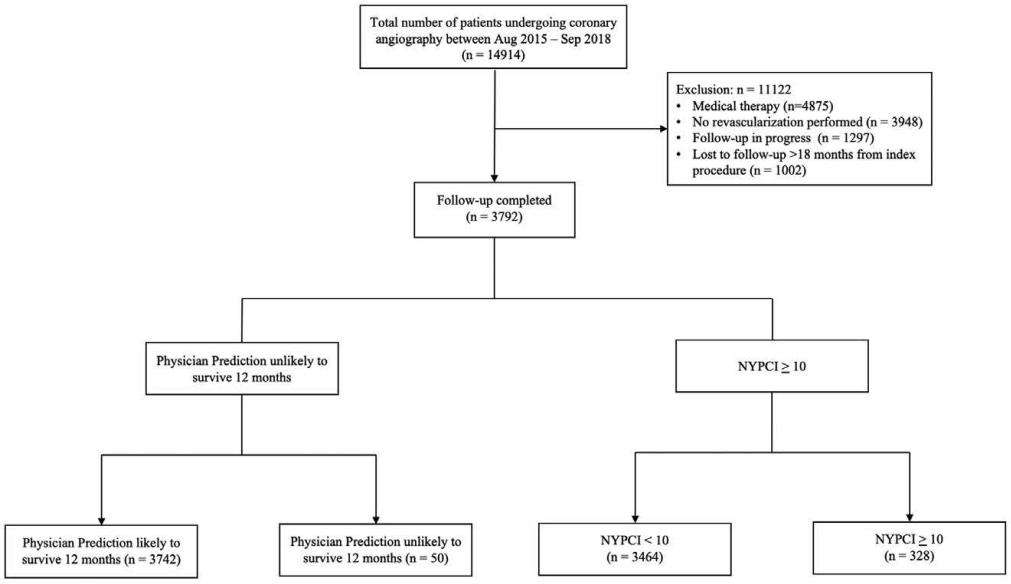
Results

Study population

Between August 2015 and September 2018, a total of 14 914 patients underwent coronary angiography and 11 222 patients were excluded as no revascularization was performed, managed by medical therapy, lost to follow-up with >18 months from the date of index procedure, or the follow-up is currently in progress (Fig. 1). A total of 3792 patients with completed follow-ups were included to evaluate PP on mortality. Based on PP, a total of 3742 patients (98.7%) were predicted to be likely to survive 12 months, and 50 patients (1.3%) were predicted to be unlikely to survive 12 months. By NYPCIRS classification, 3464 patients were stratified into the lower risk group (NYPCIRS < 10), and 328 patients had higher risk score (≥10).

Patient characteristics of the entire study population are presented in Table 1. The average age of the study population is 65.8 ± 11.8 years with 75.8% males. The cohort had an average BMI of 29.3 ± 7.4 kg/m^2 and 29.1% had type 2 diabetes. 1914 patients (50.5%) presented with acute coronary syndrome, 41 patients (1.1%) were in

Fig. 1



Flow diagram for patient selection of physician prediction of mortality.

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Table 1 Baseline characteristics

	Overall ($n = 3792$)
Age, no. (%)	65.8 ± 11.8
Sex (male), no. (%)	2875 (75.8)
BMI, kg/m^2 (mean ± SD)	29.3 ± 7.4
Hypertension, no. (%)	2360 (62.2)
Dyslipidemia, no. (%)	2316 (61.1)
Diabetes, no. (%)	1104 (29.1)
Smoking, no. (%)	
Never	2244 (59.2)
Remote (quit >1 month ago)	882 (23.3)
Active	666 (17.6)
Family history of CAD, no. (%)	486 (12.8)
Atrial fibrillation, no. (%)	233 (6.1)
Indications for angiography, no. (%)	
Acute coronary syndrome	1914 (50.5)
Staged PCI	641 (16.9)
Stable CAD	1135 (29.9)
Cardiogenic shock	41 (1.1)
ROSC	60 (1.6)
Previous history, no. (%)	
Coronary artery disease	1290 (34.0)
Percutaneous coronary intervention	1071 (28.2)
Myocardial infarction	938 (24.7)
Coronary artery bypass grafting	284 (7.5)
Peripheral artery disease	185 (4.9)
Cerebrovascular accident	147 (3.9)
Bleed	39 (1.0)
Congestive heart failure	125 (3.3)
Chronic kidney disease	105 (2.8)
Medications, no. (%)	
Aspirin	3451 (91.0)
$P2Y_{12}$ inhibitor	3455 (91.1)
ACEi/ARB	1816 (47.9)
B-blocker	2028 (53.5)
Calcium channel blocker	411 (10.8)
Statin	2929 (77.2)
Proton pump inhibitor	533 (14.1)
Nonsteroidal anti-inflammatory drug	37 (1.0)
Intervention performed, no. (%)	3163 (83.4)
NYPCI (≥10), no. (%)	328 (8.6)
Re-intervention, no. (%)	1088 (28.7)
Patient likely to live in 12 months? no. (%)	3742 (98.7)

ACEi/ARB, angiotensin converting enzyme inhibitors/angiotensin ii receptor blocker; CAD, coronary artery disease; ROSC, return of spontaneous circulation.

cardiogenic shock, and 60 patients (1.6%) presented with return of spontaneous circulation (ROSC) at the time of intervention. Finally, over 3163 patients (83.4%) had interventions performed at the time of catheterization.

Physician prediction and mortality

During a median follow-up period of 14.4 months (IQR 12.4–18.1 months), 165 patients (4.4%) had died. Patients who died were older and with greater burden of comorbidities compared to their controls and had higher proportions of both NYPCIRS≥10 and PP (Supplementary Table 1, Supplemental digital content 1, *http://links.lww.com/MCA/A395*). Interestingly, fewer interventions were performed in patients who died in 1-year follow-up. Subsequently, multivariable logistic regression was performed to evaluate factors contributing to the likelihood of receiving PCI and the strongest predictor to receive PCI was significant lesion (≥70%) with an odds ratio (OR) of 4.96 (95% CI 3.79–6.51, $P < 0.0001$). Other variables influencing PCI were renal dysfunction (OR 0.49; 95% CI 0.31–0.77, $P = 0.002$), diabetes (OR 0.66; 95% CI 0.54–0.80, $P < 0.0001$), age (OR 0.973; 95% CI 0.965–0.981, $P < 0.0001$), and BMI ≥ 40 kg/m^2 (OR 0.67; 95% CI 0.46–0.99, $P = 0.04$, Supplementary Table 2, Supplemental digital content 1, *http://links.lww.com/MCA/A395*).

PP of mortality was associated with 12-month mortality with a HR of 8.78 (95% CI 5.24–14.71, $P < 0.0001$, Fig. 2). Furthermore, after adjusting for NYPCIRS ≥10, PP remained associated with 12-month mortality with a HR of 2.92 (95% CI 1.70–5.02, $P = 0.0001$). In a post-hoc landmark analysis, PP of mortality remained associated with early and late mortality death when separated into two periods between 0 and 30 days (HR 16.4; 95% CI 8.0–33.5, $P < 0.0001$) and 30 days to 1 year (HR 4.8; 95% CI 2.1–10.9, $P < 0.0001$).

Clinical variables influencing physician prediction of mortality

Clinical factors which weighted most strongly in determining PP in our study are illustrated in Fig. 3a. Clinical presentation in the form of cardiogenic shock (OR 20.97; 95% CI 13.00–33.82, $P < 0.0001$) and ROSC (OR 4.47; 95% CI 2.41–8.28, $P < 0.0001$), as well as end organ failure in the form of liver dysfunction (OR 8.99; 95% CI 2.85–28.39, $P = 0.0002$) and renal dysfunction (OR 2.85; 95% CI 1.76–4.63, $P < 0.0001$) were associated with PP. Finally, age (OR 1.07; 95% CI 1.05–1.09, $P < 0.0001$) was associated with PP (Fig. 3a).

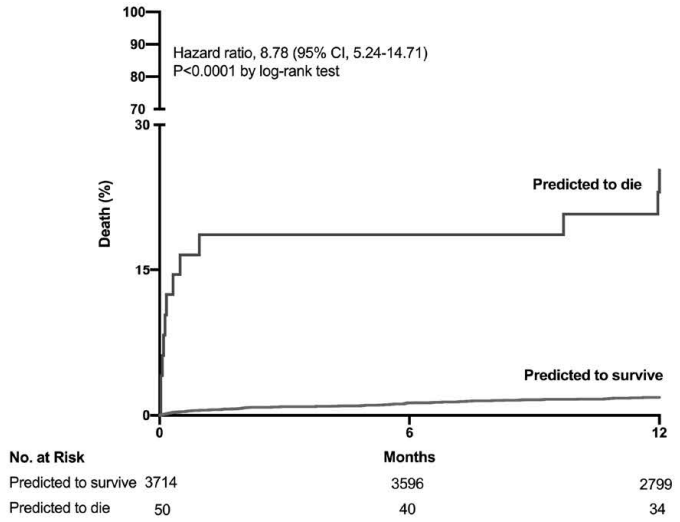
We then evaluated for independent factors which were associated with mortality (Fig. 3b). Comparatively, physicians weighted a number of factors which did not meet significance thresholds including chronic obstructive pulmonary disease (COPD) left ventricular dysfunction, BMI, and prior history of bleeding. To better elucidate which factors are over/underweighted in PP, we ratioed the OR for factors that are associated with PP to the OR for 1-year mortality. Physicians tend to overweigh the impact of prior bleeding, COPD, cardiogenic shock and liver dysfunction (Fig. 3c). Comparatively, physicians underweigh the impact of out of hospital cardiac arrest, renal dysfunction, BMI and diabetes (Fig. 3c).

Mortality accuracy comparison of physician prediction vs. New York State Percutaneous Coronary Intervention Reporting System

Diagnostic accuracy was 4.4% higher in PP compared to NYPCIRS (≥10) for mortality (95.2%, 95% CI 94.4–95.8% vs. 90.8%, 95% CI 89.8–91.7%, $P < 0.0001$). Sensitivity was lower in PP with 9.7% (95% CI 6.0–15.0%) compared to NYPCIRS with 43.6% (95% CI 36.0–52.0%, $P < 0.0001$). Specificity was higher in PP with 99.1% (95% CI 98.7–99.4%) compared to NYPCIRS with 92.9% (95% CI 92.0–94.0%, $P < 0.0001$). The positive predictive value of PP was 32.0% (95% CI 19.5–46.7%) compared to NYPCIRS with 22.0% (95% CI 18.0–27.0%, $P < 0.0001$). Negative predictive value in PP was 96.0% (95% CI 95.3–96.6%) compared to NYPCIRS with 97.3% (95% CI 96.7–97.8%, $P = 0.02$, Table 2).

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Fig. 2



Kaplan–Meier curve of physician prediction of mortality. In patients with cumulative incidence of mortality within 1-year follow-up, patients predicted to die had a higher rate of mortality (hazard ratio 8.78; 95% CI 5.24–14.71, $P < 0.0001$). Kaplan–Meier curves were generated and compared by log-rank test and hazard ratios were evaluated using the Cox proportional hazards model. $P < 0.05$ is considered statistically significant. CI, confidence interval.

Utility of physician prediction in conjunction with New York State Percutaneous Coronary Intervention Reporting System for one-year mortality

The area under the curve (AUC) for PP of mortality was 0.54 (95% CI 0.52–0.57). In comparison, NYPCIRS ≥10 had an AUC of 0.68 (95% CI 0.64–0.72) due to better sensitivity. The addition of PP of mortality to NYPCIRS modestly enhanced the AUC to 0.69 (95% CI 0.66–0.73, $P = 0.04$, Fig. 4) with an integrated discrimination improvement of 1.3% which was significant ($P = 0.03$), mainly due to a 1.2% increase in integrated sensitivity (12.3% vs. 11.1% for the new vs. old model, respectively, $P = 0.04$). With a pre-specified rate of mortality of 8% based on NYPCIRS ≥10, reclassification of patients did not improve with a net gain in reclassification proportion of 0.02 ($P = 0.11$).

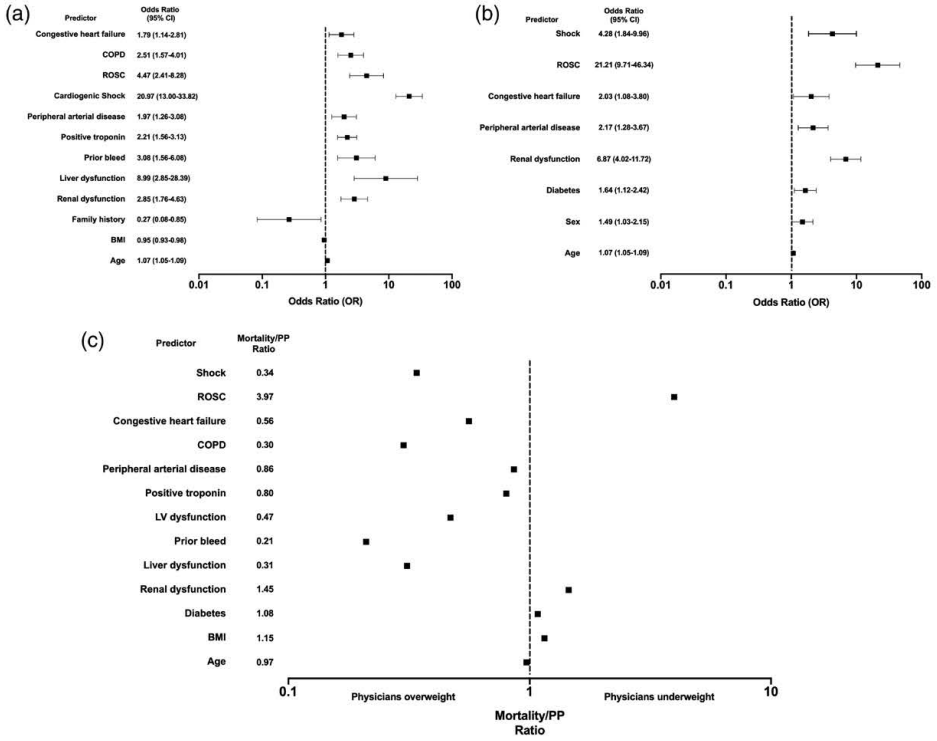
Discussion

Physician perception of mortality risk in PCI patients is a determinant of revascularization decisions in high-risk cohorts, and risk aversion may be a factor resulting in a treatment paradox [1,4]. The accuracy of physicians' risk perception in this scenario remains invalidated: our study compares PP to a validated risk score. Both PP and NYPCI scores were higher in patients who died, and PP is superior to NYPCIRS in both specificity and overall diagnostic accuracy. However, PP proved to be less sensitive and had lower positive predictive value in identifying high-risk patients. PP placed heavy weighing on mode of clinical presentation, particularly with cardiogenic shock and compared to the NYPCI in which age and severity of coronary disease were more contributory in the model. Combination of PP and NYPCIRS enhanced the AUC compared to NYPCIRS alone and improved the integrated discrimination index. Thus, our study is informative of the relative differences between PP and objective risk scoring in mortality prediction and the consequential impact that these perceptions have on patterns of treatment decision making.

A number of risk scoring tools for stratifying mortality risk in patients undergoing PCI have been externally validated in several trials [11]. However, some data has emerged suggesting that in the highest risk groups current tools may diminish in accuracy. Indeed, it has been suggested that whilst risk scoring performs well in dichotomizing patients into high and low risk broadly, 'calibration' remains difficult in individualizing specific risk outcomes for specific patients at a given point in time [12]. Furthermore, there is a dearth of data regarding the uptake and utilization of risk scoring tools by interventional cardiologists in clinical decision making during daily practice [13]. Our study helps to clarify the utility of PP as well as its limitations, and to explore its correlation with a validated tool and with clinical outcomes.

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Fig. 3



Multivariable logistic regression model to identify clinical characteristics associated with mortality. (a) Clinical characteristics associated with physician prediction of one-year mortality. (b) Clinical characteristics associated with one-year mortality. (c) Mortality to physician prediction ratio. Data are presented as odds ratios with corresponding 95% confidence intervals.

Table 2 **Diagnostic accuracy of physician prediction of mortality and NYPCIRS for assessing mortality**

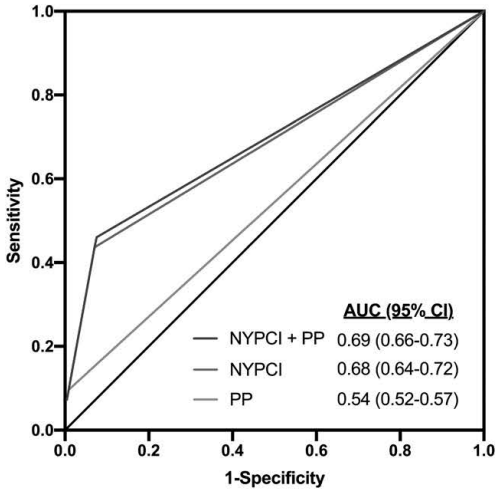
Parameter	No. (%) predicted to die ($n = 3792$)	No. (%) NYPCI ≥10 ($n = 3792$)	*P*-value[a]
Overall diagnostic accuracy, % (95% CI)	95.2 (94.4–95.8)	90.8 (89.8–91.7)	<0.0001
Specificity, % (95% CI)	99.1 (98.7–99.4)	92.9 (92.0–94.0)	<0.0001
Sensitivity, % (95% CI)	9.7 (6.0–15.0)	43.6 (36.0–52.0)	<0.0001
Negative predictive value, % (95% CI)	96.0 (95.3–96.6)	97.3 (96.7–97.8)	0.02
Positive predictive value, % (95% CI)	32.0 (19.5–46.7)	22.0 (18.0–27.0)	<0.0001
Positive likelihood ratio, no. (95% CI)	10.3 (4.6–23.1)	6.2 (4.5–8.7)	
Negative likelihood ratio, no. (95% CI)	0.91 (0.86–0.95)	0.6 (0.5–0.7)	
Diagnostic odds ratio, no. (95% CI)	11.3 (6.1–21.0)	10.2 (7.3–14.2)	

CI, confidence interval; NYPCI, New York Percutaneous Coronary Interventions Reporting System.
[a]*P*-value was based on chi-square comparison of proportions

Uniquely, we establish that a hybrid model of combining PP and NYPCIRS improves the overall diagnostic accuracy albeit modestly. Moreover, our study demonstrates that the emphasis of PP on the mode of presentation such as cardiogenic shock is likely to be an explanation for high specificity and relative merit in identifying short-term mortality as observed in the landmark analysis. Previously published data, such as that from the Canadian ACS 2 registry, supports the notion that physician perception of risk may be driven by certain acute and binary aspects of a patient's presentation such as Killip class, ECG changes, and troponin elevation, but not by other established prognostic factors or by age [3]. Our study supports the notion that age may be underappreciated

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Fig. 4



Utility of physician prediction of mortality compared to NYPCIRS for mortality. Receiver operating characteristic curve for PP with an AUC of 0.54 (95% CI 0.52–0.57) added to NYPCI with an AUC of 0.68 (95% CI 0.64–0.72) improved the AUC to 0.69 (95% CI 0.66–0.73, $P = 0.04$). AUC, area under the curve; CI, confidence interval; NYPCIRS, New York State Percutaneous Coronary Intervention Reporting System; PP, physician prediction.

in risk perception by physicians, independent of comorbid burden. In addition to age, our data demonstrates that physicians heavily weighed acute decompensation (i.e. cardiogenic shock) and multi-organ dysfunction (i.e. hepatic dysfunction and COPD). Hence, patients whose clinical presentation may not make a poor prognosis apparent, particularly in the short-term, but who's overall risk profile correlates with a poor prognosis based on age and cardiovascular risk factors, were under-identified as being at higher risk of one-year mortality by physicians.

Despite this, there are some key differences in the findings of our study with prior literature, most notably the lack of association between high-risk stratification by physicians in that study with haemodynamic instability [3]. Our study also raises the issue of a lack of sensitivity with PP. Indeed, where PP in our study demonstrates excellent specificity, the apparent emphasis on acute factors and lack of weighing of severity of coronary disease, which has a well-defined association with prognosis, is likely to account for the poor correlation of PP and low sensitivity. Indeed, in risk models such as NYPCIRS and Global Registry of Acute Coronary Events, age is consistently considered to be amongst the most important prognostic indicators available [3].

Physicians in our study were less likely to perform intervention in patients with underlying comorbidities such as renal dysfunction, type 2 diabetes, BMI ≥40 kg/m^2, and increase in age. Despite a decrease in the rate of invasive angiography and revascularization (both PCI and CABG) observed in patients with cardiogenic shock following the initiation of public reporting in the US system with self-reported data suggesting a direct causative impact, we did not observe this relationship in our dataset [14–16]. Furthermore, PP did not contribute to patients receiving intervention, potentially due to differences in Canada which reports institutional and not individual clinician characteristics.

While there remains a need for inter-institution and operator comparison and benchmarking, and a combination of outcome-based reporting as well as routine prognostication may be the key to the lack of intervention – prospectively and objectively defining the baseline risk of the cohort being treated by an institution or individual allows for better basis to form fair and valid comparisons. 'Disease based reporting' therefore may provide the key to allowing risk adjustment in adjudicating PCI outcomes. Meanwhile, risk stratification tools such as NYPCIRS should be utilized more broadly and primarily for their original purpose: as decision making aids. Ultimately, accurate risk stratification will help to avoid under-recognition of risk, potentially in elderly patients or those with severe coronary disease particularly in the longer term but treatment decisions must be individualized for specific scenarios with shared decision making involving the patient in accordance with their preferences.

Our study is not without its limitations. First, this is a single-center prospective cohort study. However, as a large regional cardiac center, we believe our findings are generalizable within Canada and other similar healthcare systems. Our model is dichotomous and does not include provision for the confidence of physicians in their prediction. Future studies could allow for more nuanced predictions and confidence ratings. Second, despite a large number of patients in our registry, a surprisingly small percentage were predicted to die at 12 months likely reflecting bias in referral patterns. Patients were referred for invasive angiography and intervention and therefore the initial referring physicians may not have referred patients in whom they felt such testing and treatment might be futile due to poor prognosis, hence resulting in selection bias in our cohort. Third, there exists the possibility of confirmation bias in our study as the patients perceived to have the worst prognosis may have been treated more conservatively, which may have increased their mortality risk even further – this may have contributed to the high PPV of PP. Lastly, our study is not immune from further selection bias as the outcome in patients who were lost to follow-up remains unknown.

Conclusion

Risk stratification in the cardiac catheterization laboratory remains a challenge for treating physicians. PP appears to be specific and accurate in prediction of mortality with

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improvements in diagnostic OR but remains inferior to NYPCIRS in both sensitivity and positive predictive value. Certain prognosticators such as cardiogenic shock and ROSC contributes heavily in PP compared with validated risk scores which weighs age and severity of CAD more heavily in the risk score. Furthermore, the combination of PP with NYPCIRS improved the c-statistic and diagnostic yield. Overall, the utility of PP with an objective risk score improves the diagnostic accuracy of mortality prediction.

Acknowledgements

The authors would like to thank the CAPITAL Research Group for all their assistance in the completion of the project.

R.G.J. was funded by the Vanier CIHR Canada Graduate Scholarship for his graduate studies.

Conflicts of interest

There are no conflicts of interest.

References

1 Narins CR, Dozier AM, Ling FS, Zareba W. The influence of public reporting of outcome data on medical decision making by physicians. *Arch Intern Med* 2005; **165**:83–87.
2 Detsky ME, Harhay MO, Bayard DF, Delman AM, Buehler AE, Kent SA, *et al.* Discriminative accuracy of physician and nurse predictions for survival and functional outcomes 6 months after an ICU admission. *JAMA* 2017; **317**:2187–2195.
3 Wu C, Camacho FT, King SB III, Walford G, Holmes DR Jr, Stamato NJ, *et al.* Risk stratification for long-term mortality after percutaneous coronary intervention. *Circ Cardiovasc Interv* 2014; **7**:80–87.
4 Fox KA, Anderson FA Jr, Dabbous OH, Steg PG, López-Sendón J, Van de Werf F, *et al.*; GRACE investigators. Intervention in acute coronary syndromes: do patients undergo intervention on the basis of their risk characteristics? The Global Registry of Acute Coronary Events (GRACE). *Heart* 2007; **93**:177–182.
5 Resnic FS, Welt FG. The public health hazards of risk avoidance associated with public reporting of risk-adjusted outcomes in coronary intervention. *J Am Coll Cardiol* 2009; **53**:825–830.
6 Hochman JS, Sleeper LA, Webb JG, Sanborn TA, White HD, Talley JD, *et al.* Early revascularization in acute myocardial infarction complicated by cardiogenic shock. *N Engl J Med* 1999; **341**:625–634.
7 Jung RG, Simard T, Di Santo P, Labinaz A, Moreland R, Duchez AC, *et al.* Performance of plasminogen activator inhibitor-1 as a biomarker in patients undergoing coronary angiography: analytical and biological considerations. *Diab Vasc Dis Res* 2019; **16**:478–482.
8 Simard T, Jung R, Labinaz A, Faraz MA, Ramirez FD, Di Santo P, *et al.* Evaluation of plasma adenosine as a marker of cardiovascular risk: analytical and biological considerations. *J Am Heart Assoc* 2019; **8**:e012228.
9 Pencina MJ, D'Agostino RB Sr, D'Agostino RB Jr, Vasan RS. Evaluating the added predictive ability of a new marker: from area under the ROC curve to reclassification and beyond. *Stat Med* 2008; **27**:157–172; discussion 207.
10 Meyers J. The %NEWSURV family of macros: an update on the survival plotting macro %NEWSURV and an introduction to expansion macros. *Pharma Sug.* 2017. May 14–17, 2017; Baltimore, MD; 2017.
11 Kunadian B, Dunning J, Das R, Roberts AP, Morley R, Turley AJ, *et al.* External validation of established risk adjustment models for procedural complications after percutaneous coronary intervention. *Heart* 2008; **94**:1012–1018.
12 Chew DP, Bhatt DL. Predicting risk in ACS. Taking the long-term view. *J Am Coll Cardiol* 2017; **70**:1858–1860.
13 Yan AT, Yan RT, Huynh T, Casanova A, Raimondo FE, Fitchett DH, *et al.*; Canadian Acute Coronary Syndrome Registry 2 Investigators. Understanding physicians' risk stratification of acute coronary syndromes: insights from the Canadian ACS 2 Registry. *Arch Intern Med* 2009; **169**:372–378.
14 Bangalore S, Guo Y, Xu J, Blecker S, Gupta N, Feit F, Hochman JS. Rates of invasive management of cardiogenic shock in New York before and after exclusion from public reporting. *JAMA Cardiol* 2016; **1**:640–647.
15 Gupta A, Gurm HS, Kirtane AJ. Upstream impact of public reporting. *Circ Cardiovasc Interv* 2019; **12**:e007878.
16 Nathan AS, Shah RM, Khatana SA, Dayoub E, Chatterjee P, Desai ND, *et al.* Effect of public reporting on the utilization of coronary angiography after out-of-hospital cardiac arrest. *Circ Cardiovasc Interv* 2019; **12**:e007564.

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Appendix G

Contrast-free optical coherence tomography: systematic evaluation of non-contrast media for intravascular assessment

Citation information:

Simard T, Motazedian P, Majeed K, Sarathy K, **Jung RG**, Ramirez FD, Feder J, Di Santo P, Parlow S, Short S, Labinaz A, Schultz C, So D, Chong AY, Le May M, Hibbert B. Contrast-free optical coherence tomography: systematic evaluation of non-contrast media for intravascular assessment. **(2020)**. *PLoS ONE.*

PLOS ONE

RESEARCH ARTICLE

Contrast-free optical coherence tomography: Systematic evaluation of non-contrast media for intravascular assessment

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Citation: Simard T, Motazedian P, Majeed K, Sarathy K, Jung RG, Feder J, et al. (2020) Contrast-free optical coherence tomography:Systematic evaluation of non-contrast media for intravascular assessment. PLoS ONE 15(8): e0237588. https://doi.org/10.1371/journal.pone.0237588

Editor: Ireneusz Grulkowski, Nicolaus Copernicus University, POLAND

Received: February 24, 2020

Accepted: July 29, 2020

Published: August 20, 2020

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: RGJ - 1 award - Vanier Research Graduate Scholarship (Canadian Institutes of Health Research) - https://vanier.gc.ca/en/home-accueil.html FDR - 2 awards - Banting Postdoctoral Fellowship (Canadian Institutes of Health Research); Detweiler Travelling Fellowship (Royal College of Physicians and Surgeons of Canada) - https://banting.fellowships-bourses.gc.ca/en/

Abstract

Background

Coronary revascularization using imaging guidance is rapidly becoming the standard of care. Intravascular optical coherence tomography uses near-infrared light to obtain high resolution intravascular images. Standard optical coherence tomography imaging technique employs iodinated contrast dye to achieve the required blood clearance during acquisition. We sought to systematically evaluate the technical performance of saline as an alternative to iodinated contrast for intravascular optical coherence tomography assessment.

Methods and results

We performed bench top optical coherence tomography analysis on nylon tubing with sequential contrast/saline dilutions to empirically derive adjustment coefficients. We then applied these coefficients *in vivo* in an established rabbit abdominal stenting model with both saline and contrast optical coherence tomography imaging. In this model, we assessed the impact of saline on both quantitative and qualitative vessel assessment. Nylon tubing assessment demonstrated a linear relationship between saline and contrast for both area and diameter. We then derived adjustment coefficients, allowing for accurate calculation of area and diameter when converting saline into both contrast and reference dimensions. *In vivo* studies confirmed reduced area with saline versus contrast [7.43 (5.67–8.36) mm^2 versus 8.2 (6.34–9.39) mm^2, $p = 0.001$] and diameter [3.08 mm versus 3.23 mm, $p = 0.001$]. Following correction, a strong relationship was achieved *in vivo* between saline and contrast in both area and diameter without compromising image quality, artefact, or strut assessment.

home-accueil.html; http://www.royalcollege.ca/rcsite/awards-grants/professional-development/detweiler-travelling-fellowship-e TS - 1 award - Vered-Beanlands Endowed Fellowship (University of Ottawa Heart Institute – Academic Medical Organization – Innovations Funding) -https://www.ottawaheart.ca/researchers/cardiovascular-research-ottawa/endowed-chairs-fellowships The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Conclusion

Saline generates reduced dimensions compared to contrast during intravascular optical coherence tomography imaging. The relationship across physiologic coronary diameters is linear and can be corrected with high fidelity. Saline does not adversely impact image quality, artefact, or strut assessment.

Introduction

Coronary angiography and percutaneous coronary intervention (PCI) are predominantly performed based on fluoroscopic and cineangiographic images for determining vessel pathology and sizing. Coronary assessment has greatly improved with the advent of intravascular imaging modalities–specifically intravascular ultrasound (IVUS) and intravascular optical coherence tomography (OCT). OCT uses near-infrared light (wavelength ~1300nm) to generate tomographic images with histological-grade resolution (10-20um). This enhanced resolution enables OCT to provide detailed intravascular assessment for thrombus, plaque morphology, intimal lesions (neointima, dissection) and stent evaluation (apposition, sizing, coverage) [1]. However, OCT requires exclusion of intraluminal blood as it causes significant attenuation of light energy owing to absorption by hemoglobin and scattering by red blood cells [2]. Hence, the use of a flushing solution to clear intraluminal blood is needed for intravascular OCT.

Contemporary FD-OCT (frequency or Fourier-domain OCT) provides pullback speeds up to 75mm/sec, enabling scanning of up to 5-7cm of an epicardial coronary vessels depending on the resolution. The first in-human studies of this technology demonstrated that this improved speed eliminates the need for proximal balloon occlusion as a single, high rate bolus injection was sufficient to exclude blood for imaging [3]. During initial development, saline was employed as a flushing medium with subsequent work demonstrating improved blood exclusion and longer imaging durations (~10 seconds) with viscous contrast media over crystalloids [3–5]. As such, viscous contrast media became the standard imaging medium used in commercial FD-OCT protocols.

Optimizing the imaging medium has garnered significant interest recently as a means of improving image quality while minimizing adverse effects. Indeed, the administration of viscous contrast media is not without risk, namely that of acute kidney injury and contrast-induced nephropathy which, though poorly understood, have been linked to long-term adverse events [6]. The side effect profile is dose-dependent, limiting the number and duration of OCT imaging runs that can be performed. This is particularly relevant in complex cases where a significant contrast load may already have been used for angiography, thus limiting OCT assessment in whom it may be the most beneficial. Accordingly, recent efforts have sought to identify alternative imaging mediums with improved side effect profiles, in particular colloids and crystalloids [7, 8]. Herein, we report the systematic evaluation of saline and saline-diluted contrast as an alternative to iodinated-contrast imaging medium for OCT-based vascular imaging.

Materials and methods

OCT and imaging media

OCT scans were acquired using a Dragonfly Imaging Catheter (St. Jude Medical, Minnesota, USA) in conjunction with the ILLUMIEN system (St. Jude Medical, Minnesota, UA). Omnipaque 300 mg/mL (GE Healthcare, NJ, USA) was used as contrast media and 0.9% sodium

chloride was used as the saline solution with varying dilutions generated via mixtures of these two agents.

Bench top model for dimensional analysis

Nylon 11 D.O.T. tubing (Freelin-Wade, Oregon, USA) with known internal diameters of 2.0/0.079, 3.0/0.118 and 4.3/0.170 mm/inches (+/- 0.08mm/0.003") were used to identify the measured differences between saline and contrast injections. We demonstrate histological and OCT cross-sections of each (Fig 1A). Varying solutions were instilled through the tubing for OCT assessment, including 100% saline, varying dilutions of saline/contrast (25%/75%, 50%/50% and 75%/25%) and 100% contrast. Representative images for a selected subset of 100% saline, 50%/50% saline/contrast and 100% contrast demonstrated in Fig 1B. For each tube size and saline/contrast combination, 15 matched images were identified and dimensional analyses were performed to assess the internal area and diameter. This process was performed for both the derivation (n = 15) and validation (n = 15) groups to generate and validate a correction factor between saline and contrast for area and diameter.

Animals and experimental protocol

All animal protocols are in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and the guidelines of the Canadian Council on Animal Care. The animal protocols were approved by the University of Ottawa Animal Care Committee. Rabbits are known to have abdominal aortas and iliac vessels similar in sizing to human coronaries [9, 10], making them a viable model for studying stent implantation and healing [11, 12]. Twenty-one male New Zealand White White Rabbits (2kg, NZW, Charles River Laboratories) were maintained in individual confinements with ample food and water supply. Animal health was monitored daily and animals were allowed regular activity periods outside of the cage. Local and systemic analgesics were employed liberally to limit any suffering and distress. Humane endpoints were continuously monitored by the animal care staff and veterinarians. Rabbits were euthanized by lethal doses of pentobarbital sodium. No mortality occurred outside the planned euthanasia or humane endpoints. Rabbits were randomly assigned by online randomization tool to undergo implantation in the abdominal aorta of either a bioresorbable vascular scaffold (BVS) (n = 11) or drug-eluting stent (DES) (n = 10). A convenience sample was employed based on available animals. Stent implantations were performed during daytime hours in a dedicated operating suite with induction by propofol and subsequent general inhaled anesthesia with vascular access achieved via femoral cut-down approach enabling placement of a 6-French femoral access sheath with subsequent closure by femoral ligation as previously reported [13, 14]. Intravenous unfractionated heparin (150U/kg) is administered intra-procedurally to prevent thrombosis, while post-stent implantation rabbits were maintained on dual anti-platelet therapy with rectal acetylsalicylic acid (ASA) 10mg/kg daily and intra-dermal clopidogrel 14mg daily to prevent stent thrombosis. OCT analyses were then performed using saline and contrast as flush solutions at two time-points–(i) immediately following stent implantation and (ii) 6-weeks post-implantation at the time of sacrifice. Three randomly selected unmatched images deemed representative of their respective scans were flagged for qualitative and quantitative evaluation in both the saline and contrast groups.

In vivo qualitative analysis

Identical de-identified images in both the saline and contrast groups underwent subjective grading to compare diagnostic quality and the presence of artifacts between the two groups.

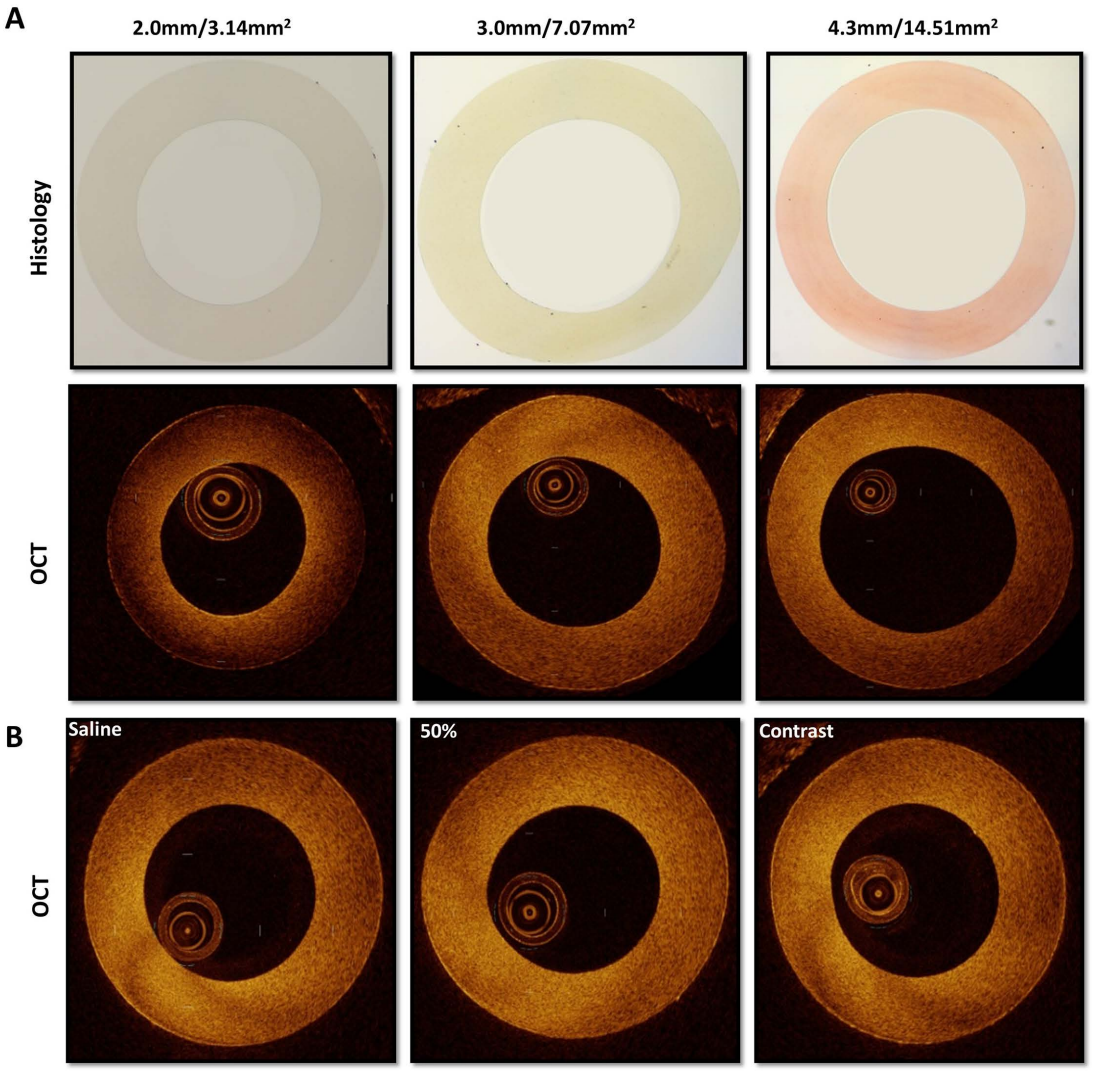


Fig 1. OCT and histological sections of nylon tubing. (A) Representative images of histological cross-sectional images of nylon tubing across physiologic size range for human coronary artery (2.0mm, 3.0mm, and 4.3mm) with corresponding OCT images of these same nylon tubes with 100% contrast flushing agent below. (B) Representative images from a select subset utilizing 100% saline, 50%/50% mixture of saline/contrast, and 100% contrast on identical nylon tubing segment.

https://doi.org/10.1371/journal.pone.0237588.g001

Scoring was completed by blinded, independent, and trained evaluators based on five predetermined criteria in a binary fashion (no = 0, yes = 1), in keeping with similar scoring systems [15]. Cumulative quality and artifact scores were assigned to each image ranging from 0 to 4. The following criteria were used to determine diagnostic quality: (1) the presence of a

clear border between lumen and luminal wall; (2) the presence of a defined border between the tunica interna and tunica media; (3) the ability to confidently identify whether stent struts were covered or uncovered; and (4) whether the overall image quality was satisfactory for diagnostic interpretation. To assess for difference in the observed artifacts between saline and contrast, four pre-established criteria were employed. Cumulative artifact scores ranging from 0 to 4 were calculated from the binary scores based on the presence or absence of the following: (1) blood swirl or speckle occupying >50% of the lumen, or the presence of thrombus; (2) sew-up artifact; (3) ghost reflections on >1 strut; and (4) saturation artifact.

In vivo quantitative analysis

Dimensional analysis similar to the bench top model was performed to assess both the luminal area and diameter with saline and contrast *in vivo*. Area and diameter were calculated via the automatic area function or manually if the software was unable to detect the luminal wall. The number of stent struts and coverage of struts were manually counted based on the presence of strut and strut reflection shadow. All measurements were completed by independent reviewers blinded to the treatment groups.

Statistical analysis

Continuous variables are reported as either mean (±SD) or median (IQR). Categorical variables are reported as either frequencies and/or percentages. All plotted relationships were linear with appropriate linear trendline fitting applied and the resulting line of best fit and R^2 displayed for each. The linear equation derived from the line of best fit was then used to derive correction formulas for each contrast concentration with validation performed in a separate cohort. Categorical variables were compared using chi-square tests, whereas continuous variables were compared using either Student t-tests or Mann-Whitney *U* tests, where appropriate.

Results

Bench top dimensional analysis

We first set out to empirically derive adjustment coefficients for dilutions of contrast on the bench top. Nylon tubing was assessed with varying concentrations of saline/contrast and the generated areas and diameters were plotted as a function of the known reference area and diameters of the tubing, demonstrating a linear relationship across all dilutions (Fig 2). As well, a progressively decreasing slope was noted as one reduced contrast content for both dimensions (Fig 2; Table 1A and 1B). The calculated linear relationships of the measured versus reference dimensions (Table 1A and 1B) were then used to derive a conversion formula for the reported contrast dilution ratios. These conversion formulas allow for the calculation of the corresponding 100% contrast dimension (Table 2A) or the reference dimension (Table 2B) for any measured dimension at a pre-defined contrast percentage (defined as X in Table 2A and 2B). We then applied these correction formulas to the raw data obtained in a separate validation cohort by plotting the corrected values versus the corresponding 100% contrast and reference values for both area and diameter. With this approach, a 1:1 relation of corrected dimension to contrast or reference dimension would indicate a reliable correction. Indeed, this was observed with validation slopes ranging from 0.98–1.03 with R^2 all greater than 0.97 (Table 2A and 2B), indicating a reliable adjustment for area and diameter across the varying flush solution compositions.

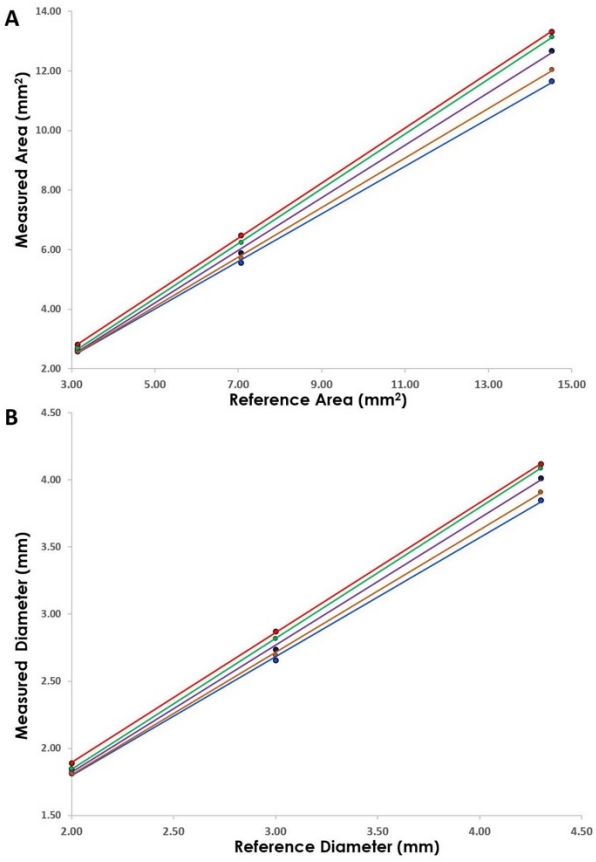


Fig 2. Measured versus reference dimensional analysis. Dimensional analysis of measured areas (A) and diameters (B) via OCT compared to reference nylon tubing dimensions. Demonstrates linear relationship with differing slopes varying by the different flush solutions employed for each. Refer to Table 1A and 1B for trendline formulas and correlation coefficients. Red– 100% contrast, Green– 75% contrast, Purple– 50% contrast, Orange– 25% contrast, Blue– 0% contrast (pure saline).

https://doi.org/10.1371/journal.pone.0237588.g002

In vivo quantitative analysis

Following our bench top assessment, we sought to assess the impact of imaging media in an *in vivo* model. This model utilizes stent implantation in the abdominal aortas of New Zealand White Rabbits with intravascular OCT assessment capable of generating histological grade imaging with both 100% saline and 100% contrast imaging media (Fig 3). Findings in this model mirrored our bench top findings with the saline cohort demonstrating a 9.4% reduction in area [7.43 (5.67–8.36) versus 8.2 (6.34–9.39 mm^2), p = 0.001] and 5% reduction in diameter [3.08 (2.68–3.26) versus 3.23 (2.84–3.46) mm, p = 0.001] in comparison to its contrast counterpart (Fig 4A and 4B).

Table 1. Dimensional analysis (A) measured versus reference area (B) measured versus reference diameter.

A	Reference (mm^2)	3.14		7.07		14.52		Measured versus Reference	
Measured (mm^2)	Contrast (%)	Mean (mm^2)	SD	Mean (mm^2)	SD	Mean (mm^2)	SD	Relation	R^2
	100	2.80	0.07	6.48	0.21	13.32	0.71	y = 0.9235x - 0.077	1
	75	2.68	0.11	6.24	0.22	13.15	1.01	y = 0.9202x - 0.2303	1
	50	2.67	0.09	5.89	0.31	12.67	0.60	y = 0.8829x - 0.203	0.9993
	25	2.59	0.08	5.71	0.41	12.05	1.05	y = 0.8331x - 0.0844	0.9997
	0	2.59	0.12	5.55	0.29	11.66	0.80	y = 0.7997x + 0.0051	0.9996
B	Reference (mm)	2.00		3.00		4.30		Measured versus Reference	
Measured (mm)	Contrast (%)	Mean (mm)	SD	Mean (mm)	SD	Mean (mm)	SD	Relation	R^2
	100	1.89	0.02	2.87	0.05	4.12	0.11	y = 0.968x - 0.0426	0.9999
	75	1.85	0.04	2.82	0.05	4.09	0.16	y = 0.9742x - 0.1036	1
	50	1.84	0.03	2.73	0.07	4.01	0.10	y = 0.946x - 0.0696	0.9993
	25	1.82	0.03	2.69	0.10	3.91	0.18	y = 0.9112x - 0.0194	0.9997
	0	1.81	0.04	2.66	0.07	3.85	0.13	y = 0.8859x + 0.0254	0.9995

https://doi.org/10.1371/journal.pone.0237588.t001

Next, we applied the bench top derived and validated dimensional correction formulas (Table 2A) to the data generated from our *in vivo* model. Specifically, we applied the correction formula for 100% saline to 100% contrast [Contrast = ((0.9194(X-0.0501))/0.7926)-0.0391] to the raw data obtained *in vivo* with 100% saline, generating corrected saline values. This correction eliminated differences between corrected saline and contrast in both area [8.52

Table 2. Conversion factors (A) Adjust measured sizing to contrast sizing (B) Adjust measured sizing to reference sizing.

Contrast (%)	Derivation (N = 45)	Validation (N = 45)	
A			
Area	Conversion formula	Slope	R^2
75	Contrast = ((0.9194(X+0.1584))/0.9063)-0.0391	1.0228	0.9952
50	Contrast = ((0.9194(X+0.1149))/0.8706)-0.0391	1.0169	0.9966
25	Contrast = ((0.9194(X+0.0928))/0.831)-0.0391	1.0029	0.992
0	Contrast = ((0.9194(X-0.0501))/0.7926)-0.0391	1.0127	0.996
Diameter	Conversion formula	Slope	R^2
75	Contrast = ((0.9708(X+0.1340))/0.9862)-0.0518	0.9824	0.996
50	Contrast = ((0.9708(X+0.1060))/0.9577)-0.0518	0.9796	0.9961
25	Contrast = ((0.9708(X+0.0255))/0.9147)-0.0518	1.0003	0.995
0	Contrast = ((0.9708(X-0.0057))/0.8928)-0.0518	0.9902	0.996
B			
Area	Conversion formula	Slope	R^2
100	Reference = (X+0.0391)/0.9194	1.0094	0.9905
75	Reference = (X+0.1584)/0.9063	1.0313	0.9834
50	Reference = (X+0.1149)/0.8706	1.0287	0.9913
25	Reference = (X+0.0928)/0.831	1.0055	0.9692
0	Reference = (X-0.0501)/0.7926	1.0184	0.9791
Diameter	Conversion formula	Slope	R^2
100	Reference = (X+0.0518)/0.9708	0.9942	0.9941
75	Reference = (X+0.1340)/0.9862	0.9757	0.9881
50	Reference = (X+0.1060)/0.9577	0.9756	0.9936
25	Reference = (X+0.0255)/0.9147	0.9924	0.9847
0	Reference = (X-0.0057)/0.8928	0.9845	0.9904

https://doi.org/10.1371/journal.pone.0237588.t002

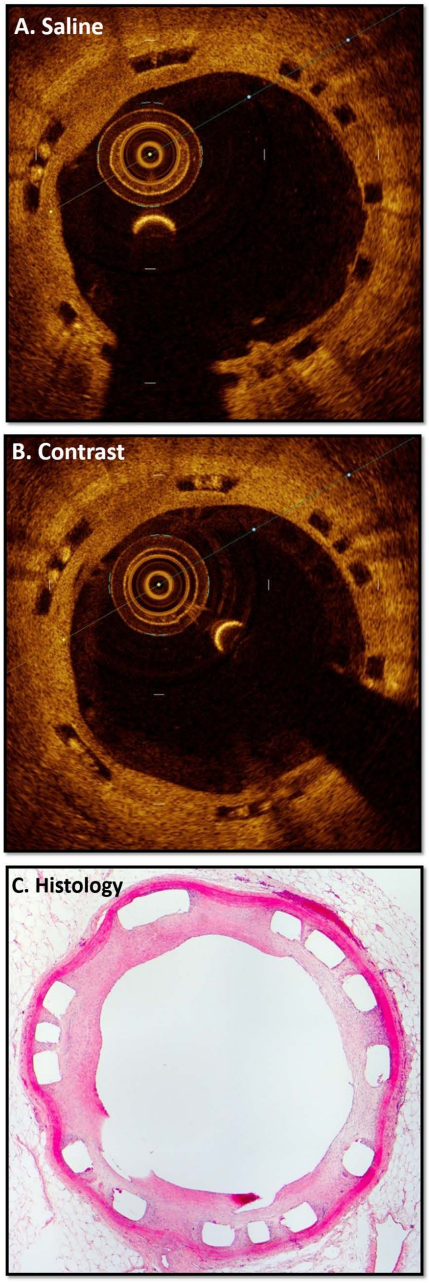

A. Saline
B. Contrast
C. Histology

Fig 3. *In vivo* comparison of OCT versus traditional histology. Images of identical rabbit arterial segment with scaffold in situ and subsequent arterial healing and neointima formation. Specifically note of robust virtual histology with OCT utilizing both 100% saline (A) and 100% contrast (B) flushing agents when compared to traditional histology with haematoxylin and eosin (H&E) staining (C).

https://doi.org/10.1371/journal.pone.0237588.g003

(6.48–9.60) vs 8.20 (6.34–9.39) mm^2, 9.4% to 3.9% absolute difference, p = 0.35, Fig 4A) and diameter [3.29 (2.86–3.49) vs 3.23 (2.84–3.46) mm, 5% to 1.9% absolute difference, p = 0.40, Fig 4B). Similarly, when the corrected saline values were plotted as a function of the measured contrast values, they demonstrated a strong 1:1 linear relationship indicating robust correction for both area (slope = 1.06, R^2 = 0.89, Fig 4E) and diameter (slope = 1.03, R^2 = 0.87, Fig 4F) when compared to uncorrected dimensions with saline versus contrast (Fig 4C and 4D).

Last, we used the *in vivo* images to quantify stent struts and arterial healing post stent implantation. The ability to identify stent struts and describe their appearance (i.e. exposed or covered) is of particular importance to assessing stent deployment and pathology. We did not observe any differences between saline or contrast with regards to the total number of stent struts, exposed struts, or covered struts (Table 3). Furthermore, neointima quantification by OCT imaging with saline and contrast similarly demonstrated no differences between groups (32.3+/-6.46 versus 30.3+/-5.56%, p = 0.25) (Table 3).

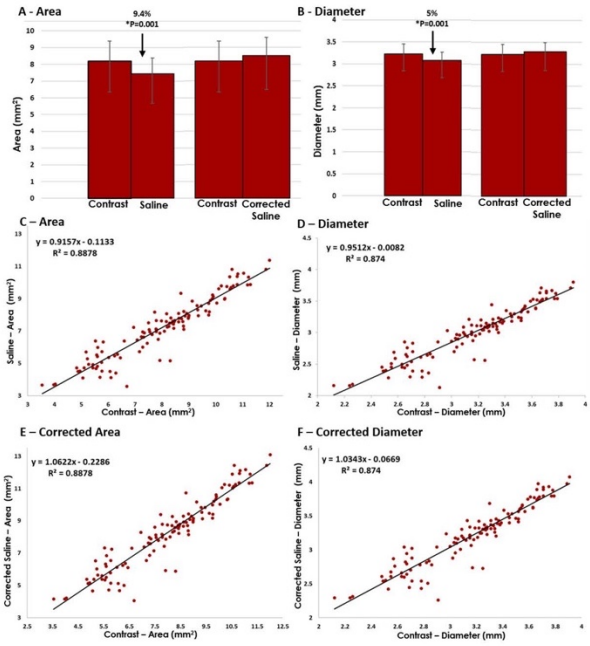


Fig 4. *In vivo* quantitative dimensional analysis. Dimensional analysis of area (A) and diameter (B) in rabbit model of intravascular OCT. Note of a significant 9.4% reduced area and 5% reduced diameter in the saline versus contrast cohorts. Following application of empirically derived correction factors no further significant difference between either cohorts. Graphical depiction of individual data points for saline versus contrast assessments for both area (C) and diameter (D) demonstrating linear relationship with slopes of 0.9157 and 0.9512 respectively. Following application of empiric correction factors note of augmented slope to 1.0622 and 1.0343 respectively for area (E) and diameter (F).

https://doi.org/10.1371/journal.pone.0237588.g004

Table 3. *In vivo* quantitative stent analysis.

	Saline		Contrast		
	Mean	SD	Mean	SD	p
Strut Assessment (N)	117		117		
Exposed struts	5.81	6.63	5.44	6.06	0.92
Covered struts	6.29	6.78	6.79	6.74	0.55
Total strut number	12.10	4.42	12.23	3.68	0.9
Neointima assessment (N)	25		25		
Neointima size (%)	32.3	6.46	30.3	5.56	0.25

https://doi.org/10.1371/journal.pone.0237588.t003

In vivo qualitative analysis

Based on the 4-point quality scoring system, all *in vivo* images for saline (n = 117) and contrast (n = 117) were assessed by independent reviewers for each quality component to generate a summative overall quality score (Table 4). Ultimately, no significant differences between any of the individual quality components, or in the overall quality scores were observed. Similarly, independent reviewers evaluated both the saline (n = 117) and contrast (n = 117) cohorts for the presence of common artefacts known to compromise intravascular OCT imaging (Table 5; examples are demonstrated in Fig 5). Overall, no significant differences in any of the artefacts screened for were demonstrated between either groups. Both saline and contrast yielded image quality on par with histological-grade images, with corresponding histological sections demonstrating striking similarities (Fig 3).

Discussion

Intravascular OCT provides histological-grade intracoronary assessment, but requires clearance of intraluminal blood via flushing with viscous contrast solution, which carries risks [16]. Preliminary work has investigated the utility of varying flush solutions including dextran and saline with promising results [17, 18]. However, a thorough preclinical assessment of the qualitative and quantitative properties has yet to be reported. Herein, we provide empirically-derived and validated correction formulas for dimensional analysis for saline-based imaging. Moreover, we demonstrate the utility of these correction formulas in an *in vivo* setting for dimensional analysis while also demonstrating no significant impact on quantitative and qualitative stent assessments in a preclinical rabbit model.

Dimensional analysis

Our bench top and *in vivo* models enable assessment of the impact of the contrast-content of imaging media on measured intravascular areas and diameters. Both models demonstrate a significant difference with respect to the dimensions reported between saline and contrast in

Table 4. *In vivo* qualitative analysis–quality score.

	Saline	%/SD	Contrast	%/SD	p
N	**117**		**117**		
Luminal border	109	93.1	108	92.3	1.00
Intima/media border	43	36.8	37	31.6	0.49
Stent strut	100	85.4	95	81.2	0.48
Diagnostic quality	99	84.6	93	79.5	0.39
Overall score	3.0	1	2.85	1.06	0.06

https://doi.org/10.1371/journal.pone.0237588.t004

Table 5. *In vivo* qualitative analysis–artefact score.

	Saline	%	Contrast	%	p
Number	**117**		**117**		
Blood score	23	19.7	12	10.3	0.067
Sew-up	0	0.0	1	0.9	1.000
Ghost-strut	0	0.0	0	0.0	-
Saturation	0	0.0	0	0.0	-

https://doi.org/10.1371/journal.pone.0237588.t005

keeping with previous reports [19]. The observed differences in dimensions are likely related to the varying refractive indices between saline (1.33) and contrast (1.44), with most commercial systems employing a refractive index of 1.4 for dimensional calculations [19]. In our bench top model we similarly noted a 10% difference in sizing that, once corrected, yielded no difference between the two groups (4%). Similarly, when assessed in the *in vivo* setting no differences in dimensions remained following correction, with only a 4% difference in area and 2% difference in diameter remaining. This is in keeping with previous work in a swine model that reported a variance in area of 18% that was reduced to 2.9% with correction based upon the refractive index of the solution [19]. Our empirically-derived correction factors thus enable robust adjustment for a broad range of contrast dilutions across a physiologically-relevant range of luminal diameters.

Qualitative and quantitative assessments in diagnostic quality

In addition to vessel sizing, OCT is commonly performed to clarify unclear anatomy, assess for dissections, and thrombus, and/or plaque morphology. Thus, acquiring a diagnostic image to permit assessment of the vessel architecture is paramount for imaging performance. Accordingly, we assessed qualitative measures in an established rabbit stenting model by performing a standardized assessment of a reviewers' ability to identify key vessel architectural features and artefact presence [15]. In our study, we found no significant differences between saline and contrast in any of the individual quality components, nor in the overall sum quality score suggesting that saline performs comparably to contrast in our system. Similarly, reviewers assessed for the presence of artefacts and did not identify any differences between saline and contrast, though there may be a suggestion of more blood artefact in the saline cohort. This is most likely related to reduced flushing efficacy with saline owing to its >10-fold lower viscosity than iodinated contrast [19, 20]. Indeed, the dynamics of flushing has been explored previously with viscosity, flow rate and flush duration identified as the predominant factors impacting blood displacement [19]. We employed a standardized flushing protocol with a rate sufficient to clear blood and to initiate auto-triggering of the OCT run. This rate was then maintained for a fixed duration for each run; therefore, any suggested any variance in image quality would be most likely related to differing viscosities of the two agents. To overcome the reduced viscosity, one could improve blood clearance by either increasing the flush duration and/or flow rate [19]. Moreover, this effect is likely magnified in the rabbit model as flushing is performed in a retrograde fashion against aortic blood flow as opposed to an antegrade approach in human coronaries.

Study limitations

Our work is not without limitations. First, our bench top model is based on standardized internal diameters, which, while stringently manufactured and controlled for, still report variances in sizing of ±0.08mm/0.003". However, this variance should minimally impact our adjustment

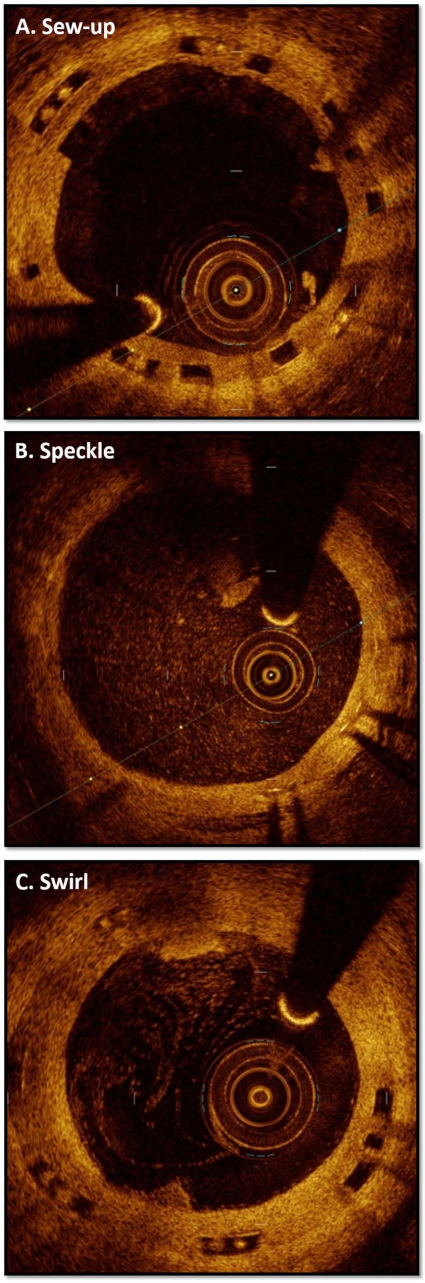

A. Sew-up
B. Speckle
C. Swirl

Fig 5. Artefacts with intravascular OCT. Examples of common artefacts with intravascular OCT including sew-up artefact (A) related to motion, as well as speckle (B) and swirl (C) artefacts related to insufficient intravascular blood flushing.

https://doi.org/10.1371/journal.pone.0237588.g005

coefficients, given its low magnitude and equal distribution in both groups. Second, the importance of arterial blood clearance is paramount for robust image production. The abdominal aorta of rabbits is an established model for assessment of stent implantation and healing [11, 12]. However, while it provides a comparable physiologic system and dimensions to human coronary arteries it is less complex than the human coronary tree. Clinical performance in human coronaries would be needed to corroborate our findings and to optimize and validate saline as a dedicated contrast agent for OCT imaging during diagnostic and interventional coronary procedures. While anecdotal use of saline is reported, varying parameters such as volumes, injection parameters and coronary complexity would need to be standardized for evaluation.

Conclusions

In summary, saline generates reduced dimensions in a linear fashion, enabling robust correction to contrast values. Our model suggests no significant difference in image quality with saline flushing on account of its reduced viscosity, while any differences could likely be mitigated by varying the flush rate and/or duration. Clinical studies investigating the efficacy of saline as a contrast agent for OCT imaging are warranted.

Supporting information

S1 Dataset.
(XLSX)

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References

1. Bezerra HG, Attizzani GF, Sirbu V et al. Optical coherence tomography versus intravascular ultrasound to evaluate coronary artery disease and percutaneous coronary intervention. JACC Cardiovasc Interv 2013; 6:228–36. https://doi.org/10.1016/j.jcin.2012.09.017 PMID: 23517833
2. Terashima M, Kaneda H, Suzuki T. The Role of Optical Coherence Tomography in Coronary Intervention. The Korean Journal of Internal Medicine 2012; 27:1–12. https://doi.org/10.3904/kjim.2012.27.1.1 PMID: 22403493
3. Tearney GJ, Waxman S, Shishkov M et al. Three-Dimensional Coronary Artery Microscopy by Intracoronary Optical Frequency Domain Imaging. JACC Cardiovascular imaging 2008; 1:752–761. https://doi.org/10.1016/j.jcmg.2008.06.007 PMID: 19356512
4. Prati F, Jenkins MW, Di Giorgio A, Rollins AM. Intracoronary optical coherence tomography, basic theory and image acquisition techniques. The international journal of cardiovascular imaging 2011; 27:251–258. https://doi.org/10.1007/s10554-011-9798-1 PMID: 21327912
5. Prati F, Cera M, Ramazzotti V, Imola F, Giudice R, Albertucci M. Safety and feasibility of a new non-occlusive technique for facilitated intracoronary optical coherence tomography (OCT) acquisition in various clinical and anatomical scenarios. EuroIntervention 2007; 3:365–70. https://doi.org/10.4244/eijv3i3a66 PMID: 19737719
6. Andreucci M, Solomon R, Tasanarong A. Side Effects of Radiographic Contrast Media: Pathogenesis, Risk Factors, and Prevention. BioMed Research International 2014; 2014:741018. https://doi.org/10.1155/2014/741018 PMID: 24895606
7. Kataiwa H, Tanaka A, Kitabata H et al. Head to head comparison between the conventional balloon occlusion method and the non-occlusion method for optical coherence tomography. Int J Cardiol 2011; 146:186–90. https://doi.org/10.1016/j.ijcard.2009.06.059 PMID: 19664829
8. Prati F, Cera M, Ramazzotti V et al. From bench to bedside: a novel technique of acquiring OCT images. Circ J 2008; 72:839–43. https://doi.org/10.1253/circj.72.839 PMID: 18441468
9. Byrom MJ, Bannon PG, White GH, Ng MKC. Animal models for the assessment of novel vascular conduits. Journal of Vascular Surgery 2010; 52:176–195. https://doi.org/10.1016/j.jvs.2009.10.080 PMID: 20299181
10. Simard T, Hibbert B, Ramirez FD, Froeschl M, Chen YX, O'Brien ER. The evolution of coronary stents: a brief review. Can J Cardiol 2014; 30:35–45. https://doi.org/10.1016/j.cjca.2013.09.012 PMID: 24286961
11. Schwartz RS, Chronos NA, Virmani R. Preclinical restenosis models and drug-eluting stents: Still important, still much to learn. Journal of the American College of Cardiology 2004; 44:1373–1385. https://doi.org/10.1016/j.jacc.2004.04.060 PMID: 15464316
12. Suzuki Y, Yeung AC, Ikeno F. The Pre-Clinical Animal Model in the Translational Research of Interventional Cardiology. JACC: Cardiovascular Interventions 2009; 2:373–383. https://doi.org/10.1016/j.jcin.2009.03.004 PMID: 19463458
13. Ma X, Hibbert B, Dhaliwal B et al. Delayed re-endothelialization with rapamycin-coated stents is rescued by the addition of a glycogen synthase kinase-3beta inhibitor. Cardiovasc Res 2010; 86:338–45. https://doi.org/10.1093/cvr/cvq047 PMID: 20167573

14. Ma X, Hibbert B, McNulty M et al. Heat shock protein 27 attenuates neointima formation and accelerates reendothelialization after arterial injury and stent implantation: importance of vascular endothelial growth factor up-regulation. Faseb j 2014; 28:594–602. https://doi.org/10.1096/fj.13-230417 PMID: 24142570

15. Huang Y, Gangaputra S, Lee KE et al. Signal quality assessment of retinal optical coherence tomography images. Invest Ophthalmol Vis Sci 2012; 53:2133–41. https://doi.org/10.1167/iovs.11-8755 PMID: 22427567

16. Meissner OA, Rieber J, Babaryka G et al. Intravascular optical coherence tomography: comparison with histopathology in atherosclerotic peripheral artery specimens. Journal of vascular and interventional radiology: JVIR 2006; 17:343–9. https://doi.org/10.1097/01.RVI.0000195324.52104.00 PMID: 16517781

17. Kendrick DE, Allemang MT, Gosling AF et al. Dextran or Saline Can Replace Contrast for Intravascular Optical Coherence Tomography in Lower Extremity Arteries. Journal of Endovascular Therapy 2016; 23:723–730. https://doi.org/10.1177/1526602816657392 PMID: 27385151

18. Secco GG, Grattoni C, Parisi R et al. Saline vs contrast infusion during optical coherence tomography imaging of peripheral percutaneous intervention. Int J Cardiol 2014; 172:246–8. https://doi.org/10.1016/j.ijcard.2013.12.288 PMID: 24447736

19. Suter MJ, Kashiwagi M, Gallagher KA et al. Optimizing flushing parameters in intracoronary optical coherence tomography: an in vivo swine study. Int J Cardiovasc Imaging 2015; 31:1097–106. https://doi.org/10.1007/s10554-015-0668-0 PMID: 25922149

20. Berteau C, Filipe-Santos O, Wang T, Rojas HE, Granger C, Schwarzenbach F. Evaluation of the impact of viscosity, injection volume, and injection flow rate on subcutaneous injection tolerance. Med Devices (Auckl) 2015; 8:473–84.

Appendix H

Commentary: geographic variation in the statin trials – underrepresentation of Asian populations

Citation information:

Jung RG, Di Santo P, Hibbert B. Commentary: geographic variation in the statin trials – underrepresentation of Asian populations. (**2020**). *International Journal of Cardiology*.

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Commentary: Geographic variation in the statin trials - underrepresentation of Asian populations

Author: Richard G. Jung,Pietro Di Santo,Benjamin Hibbert

Publication: International Journal of Cardiology

Publisher: Elsevier

Date: 1 October 2020

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International Journal of Cardiology 316 (2020) 47–48

Contents lists available at ScienceDirect

International Journal of Cardiology

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Editorial

Commentary: Geographic variation in the statin trials - underrepresentation of Asian populations

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1. Introduction

Statin therapy remains a cornerstone of primary and secondary prevention of atherosclerotic cardiovascular disease (**ASCVD**) for reduction of major adverse cardiovascular events [1]. Randomized controlled trials (**RCT**) are performed to evaluate treatment efficacy and safety with global participation in order to apply the results to a broad population. Despite multi-center RCTs spanning across multiple continents, a wide ethnic variation of response and dosage to statins currently exists [2,3]. Furthermore, multi-center RCTs underrepresent lower-income countries which affects the general applicability of results. In the systematic review performed by *Adams* et al., they sought to evaluate whether differences in statin effect are due to underrepresentation of certain geographic regions in RCTs [10].

2. Geographic and ethnic variation in statins

In the systematic review evaluating variations in statin trials, *Adams* et al. identified 20 RCTs with a total of 138,612 participants and report that 73.9% participants originate from North America or Europe [10]. The authors highlight a significant limitation of multi-center RCTs in statin trials, where enrolment from China and India represented only 4.0% of the studied population despite the two countries comprising over 35% of the global population with disparate rates of ASCVD. They highlight the lack of representation from lower-income regions which remain at high-risk for ASCVD and postulate that underlying economic, market, and governmental forces drives the clinical trial research landscape.

Indeed, few patients from each country are typically enrolled into RCTs which highlights a major limitation in multi-region RCTs where patients comprising mainly of one region drives the outcome and subsequent guidelines may not be applicable to other regions around the world. Furthermore, geographical variations in statin-use and compliance is closely associated with regional income and proximity to academic medical centers [4]. Ethnic and sex variations have also been observed in statin trials with heterogeneous responses to statin dosage and LDL reduction [5,6]. Ethnic variation in LDL reduction to statins has been partially explained due to genetic and pharmacokinetic variations [5]. Moreover, ethnic variations in statin-use may partially be due to differences in drug adherence mediated by multiple socioeconomic factors, access to care, and patient preferences [7]. The results of the systematic review are thought-provoking in that Asian countries were underrepresented in trials examining statins which limits generalizability.

3. Geographic variation in trial enrolment influencing differences in regional outcomes

Discrepancies in geographic regional recruitment remains pervasive and is not only limited to statins. Multi-center RCTs enrol varying number of patients across multiple continents with underlying differences in baseline characteristics and standard of care [8]. Typically, subgroup analysis to determine differences by geographical region is conducted using statistical test of interaction but is often plagued by low statistical power and *post-hoc* multiplicity of hypotheses and stratification of countries into regions [9]. Significant evidence of heterogeneity between geographic regions often occurs due to chance and are hypothesis-generating, but sometimes has underlying biological plausibility and warrants further investigation [8]. The observed regional differences in subgroup analyses may be due to differences in patient selection such as ethnic variation or environmental differences, standard of care, and reporting patterns in outcomes [9]. Indeed, these geographic variations for treatment efficacy and safety are not ignored in trial designs as governmental agencies such as the Food and Drug Administration requires that pivotal trials recruit a certain number of patients from the United States.

In conclusion, geographical variations in statin trials are a microcosm of underrepresentation of non-Western world regions. Future RCTs or prospective studies may seek to balance recruitment of patients from multiple regions to permit greater applicability while considering patient selection, standard-of-care, and outcome reporting to account for geographic variation in clinical trials.

DOI of original article: https://doi.org/10.1016/j.ijcard.2020.07.015.
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https://doi.org/10.1016/j.ijcard.2020.07.023
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Declaration of Competing Interest

The authors report no relationships that could be construed as a conflict of interest for the completion of this work.

References

[1] S.M. Grundy, N.J. Stone, A.L. Bailey, C. Beam, K.K. Birtcher, R.S. Blumenthal, et al., 2018 AHA/ACC/AACVPR/AAPA/ABC/ACPM/ADA/AGS/APhA/ASPC/NLA/PCNA guideline on the management of blood cholesterol: a report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines, Circulation 139 (2019) e1082–e1143.

[2] E. Lee, S. Ryan, B. Birmingham, J. Zalikowski, R. March, H. Ambrose, et al., Rosuvastatin pharmacokinetics and pharmacogenetics in white and Asian subjects residing in the same environment, Clin. Pharmacol. Ther. 78 (2005) 330–341.

[3] H. Nakamura, K. Arakawa, H. Itakura, A. Kitabatake, Y. Goto, T. Toyota, et al., Primary prevention of cardiovascular disease with pravastatin in Japan (MEGA study): a prospective randomised controlled trial, Lancet 368 (2006) 1155–1163.

[4] V. Bittner, L.D. Colantonio, Y. Dai, M. Woodward, M.T. Mefford, R.S. Rosenson, et al., Association of region and hospital and patient characteristics with use of high-intensity statins after myocardial infarction among medicare beneficiaries, JAMA Cardiol. 4 (2019) 865–872.

[5] R. Naito, K. Miyauchi, H. Daida, Racial differences in the cholesterol-lowering effect of statin, J. Atheroscler. Thromb. 24 (2017) 19–25.

[6] Y.F. Li, Q.Z. Feng, W.Q. Gao, X.J. Zhang, Y. Huang, Y.D. Chen, The difference between Asian and Western in the effect of LDL-C lowering therapy on coronary atherosclerotic plaque: a meta-analysis report, BMC Cardiovasc. Disord. 15 (2015) 6.

[7] M.G. Nanna, A.M. Navar, P. Zakroysky, Q. Xiang, A.C. Goldberg, J. Robinson, et al., Association of patient perceptions of cardiovascular risk and beliefs on statin drugs with racial differences in statin use: insights from the patient and provider assessment of lipid management registry, JAMA Cardiol. 3 (2018) 739–748.

[8] S. Yusuf, J. Wittes, Interpreting geographic variations in results of randomized, controlled trials, N. Engl. J. Med. 375 (2016) 2263–2271.

[9] S. Pocock, G. Calvo, J. Marrugat, K. Prasad, L. Tavazzi, L. Wallentin, et al., International differences in treatment effect: do they really exist and why?†, Eur. Heart J. 34 (2013) 1846–1852.

[10] C Adams, K Singh, Geographic variation in the statin trials: Underrepresentation of Asian populations, International Journal of Cardiology (2020), https://doi.org/10.1016/j.ijcard.2020.07.015.

Appendix I

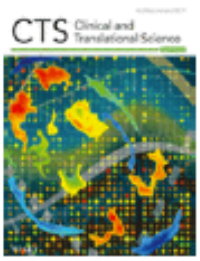
Performance of plasma adenosine as a biomarker for predicting cardiovascular risk

Citation information:

Jung RG*, Simard T, Di Santo P, Ramirez FD, Labinaz A, Gaudet C, Motazedian P, Parlow S, Joseph J, Moreland R, Marbach JA, Boland P, Promislow S, Russo JJ, Chong AY, So D, Labinaz M, Le May M, Hibbert B. Performance of plasma adenosine as a biomarker for predicting cardiovascular risk. (**2020**). *Clinical and Translational Science*.

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Performance of Plasma Adenosine as a Biomarker for Predicting Cardiovascular Risk

Author: Trevor Simard, Richard G. Jung, Pietro Di Santo, et al

Publication: Clinical and Translational Science

Publisher: John Wiley and Sons

Date: Dec 2, 2020

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ARTICLE

Performance of Plasma Adenosine as a Biomarker for Predicting Cardiovascular Risk

Trevor Simard[1,2,3,†], Richard G. Jung[1,2,†], Pietro Di Santo[1], F. Daniel Ramirez[1,4,5], Alisha Labinaz[1], Chantal Gaudet[1], Pouya Motazedian[1,6], Simon Parlow[1], Joanne Joseph[1], Robert Moreland[1], Jeffrey Marbach[1], Paul Boland[1], Steven Promislow[1], Juan J. Russo[1], Aun-Yeong Chong[1], Derek So[1], Michael Froeschl[1], Michel Le May[1] and Benjamin Hibbert[1,2,*]

Adenosine boasts promising preclinical and clinical data supporting a vital role in modulating vascular homeostasis. Its widespread use as a diagnostic and therapeutic agent have been limited by its short half-life and complex biology, though adenosine-modulators have shown promise in improving vascular healing. Moreover, circulating adenosine has shown promise in predicting cardiovascular (CV) events. We sought to delineate whether circulating plasma adenosine levels predict CV events in patients undergoing invasive assessment for coronary artery disease. Patients undergoing invasive angiography had clinical data prospectively recorded in the Cardiovascular and Percutaneous CIInical TriALs (CAPITAL) revascularization registry and blood samples collected in the CAPITAL Biobank from which adenosine levels were quantified. Tertile-based analysis was used to assess prediction of major adverse cardiovascular events (MACE; composite of death, myocardial infarction, unplanned revascularization, and cerebrovascular accident). Secondary analyses included MACE subgroups, clinical subgroups and adenosine levels. There were 1,815 patients undergoing angiography who had blood collected with adenosine quantified in 1,323. Of those quantified, 51.0% were revascularized and 7.3% experienced MACE in 12 months of follow-up. Tertile-based analysis failed to demonstrate any stratification of MACE rates (log rank, $P = 0.83$), when comparing low-to-middle (hazard ratio (HR) 1.10, 95% confidence interval (CI) 0.68–1.78, $P = 0.70$) or low-to-high adenosine tertiles (HR 0.95, 95% CI 0.56–1.57, $P = 0.84$). In adjusted analysis, adenosine similarly failed to predict MACE. Finally, adenosine did not predict outcomes in patients with acute coronary syndrome nor in those revascularized or treated medically. Plasma adenosine levels do not predict subsequent CV outcomes or aid in patient risk stratification.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
☑ Adenosine demonstrates promising preclinical and clinical data supporting its role as a vasculoprotective agent with limited data suggesting it may serve as a marker of clinical outcomes.

WHAT QUESTION DID THIS STUDY ADDRESS?
☑ We sought to definitively establish the role of circulating adenosine as a predictor of cardiovascular (CV) events in patients undergoing invasive assessment for coronary artery disease.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?
☑ Systemic adenosine levels do not predict CV outcomes or aid in CV risk stratification.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?
☑ Although risk stratification using systemic adenosine levels are not clinically useful, this does not preclude the potential of tissue-level adenosine differences nor the role of adenosine modulation for therapeutic benefit.

Adenosine serves as a crucial regulatory molecule in both intracellular and extracellular processes.[1] Adenosine homeostasis is closely regulated by an ongoing balance of production, degradation, and transport.[2,3] Adenosine signaling is complex with four primary adenosine receptors (1, 2A, 2B, and 3) facilitating extracellular signaling. These receptors are found on a multitude of vascular cells with differing responses based upon the cell and receptor

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Received: May 20, 2020; accepted: July 23, 2020. doi:10.1111/cts.12886

subtypes. Accordingly, adenosine signaling has been implicated in vascular homeostasis with considerable preclinical data supporting its role in vasculoprotection.[1]

Therapeutically, adenosine is limited by its relative instability and short half-life. As a hyperemic agent, it has found limited use in coronary flow assessment,[4] although dipyridamole is more commonly used to augment adenosine levels given its improved stability.[5] Dedicated molecules specifically targeting adenosine receptors of interest are also in use to further optimize the intended vs. unintended effects of adenosine modulation.[6] Indeed, adjunctive agents that augment adenosine levels have demonstrated promising results. Dipyridamole, augmenting adenosine through equilibrative nucleoside transporter-1 (ENT-1) inhibition, has demonstrated improved vascular patency and reduced restenosis in preclinical and clinical studies.[1] Similarly, cilostazol modestly augments adenosine levels in addition to cyclic adenosine monophosphate,[7] yielding reduced restenosis following coronary stenting.[8] Last, ticagrelor, a $P2Y_{12}$ receptor inhibitor, demonstrates improved clinical outcomes aside from its antiplatelet effects proposed to be related to pleiotropic effects via augmentation of adenosine[9] (via ENT-1 inhibition)[10] and possibly cyclic adenosine monophosphate.[11] However, the precise impact of ticagrelor on circulating adenosine levels in clinical trials remains the subject of debate.[12,13] Taken together, preclinical and clinical data supports that adenosine augmentation may portend vasculoprotective effects and thereby improve cardiovascular (CV) outcomes.

Diagnostically, circulating adenosine levels have not been widely adopted owing in part to the technical challenges with its quantification and notable biological variability.[14] Despite this, small series have suggested a role for adenosine levels in risk stratifying CV outcomes. Subjects with coronary artery disease (CAD) and genetically reduced adenosine deaminase (AMPD1) activity, theoretically increasing adenosine levels, have demonstrated improved CV survival.[15] Similarly, we previously reported diminished adenosine levels in those with advanced age and obstructive CAD, although no association with established risk factors was noted.[14] However, following revascularization, patients with AMPD1 did not demonstrate improved CV outcomes.[16] This study was limited by its small size, nonconventional revascularization strategies, and lack of difference in adenosine levels between the cohorts—supporting the challenges of utilizing adenosine as a biomarker.[16] Patients post-revascularization are at elevated risk of recurrent CV events,[17] and exogenous adenosine has shown promise in improving vascular healing in preclinical models.[18] Given the uncertainty of the utility of adenosine as a biomarker, we sought to determine whether circulating adenosine levels are predictive of future CV events in patients undergoing invasive assessment for CAD.

METHODS

Adenosine sample collection and quantification

Adenosine samples were collected at the time of invasive angiography as previously described.[14] Briefly, blood samples were collected via the plastic arterial access sheath (Terumo Medical, Somerset, NJ) or, rarely, if required by peripheral venipuncture. Samples were collected in prefilled Greiner BioOne Vacuette tubes containing 2 mL of ice-cold stop solution as reported.[14] Hemolyzed samples were excluded.[19] Samples were centrifuged and stored at –80°C to await processing. Samples were then processed via the Waters Oasis MCX (Mixed-mode, strong Cation-eXchange) columns and transferred for high-performance liquid chromatography analysis on a Waters Alliance E2695 separating module system with sample quantification by Waters 2489 UV/visible detector at 260 nM as previously reported.[14] This methodology underwent thorough development and validation with ongoing quality-of-care metrics to ensure robust adenosine quantification as described.[14]

Population and patient outcomes

The University of Ottawa Heart Institute provides sole revascularization services to over 1.2 million people.[20,21] The Cardiovascular and Percutaneous CIInical TriALs (CAPITAL) revascularization registry is a web-based registry that prospectively indexes data on patients undergoing coronary angiography. Baseline demographics, CV risk factors and definitions have been previously described.[14] Patients undergo follow-up assessment in the year following their angiography where clinical outcomes in the year following revascularization are recorded in the registry. Outcomes were prospectively recorded by individuals blinded to adenosine levels. Myocardial infarction (MI) was recorded according to the universal definition of MI or clinical diagnosis of MI by treating physicians.[22] Repeat unplanned revascularization included patients undergoing repeat coronary intervention not planned following their index procedure and included both target vessel and non-target vessel revascularization. Death included both cardiac and noncardiac death. Stroke included both ischemic or hemorrhagic events as diagnosed by the treating neurologist or on cross-sectional imaging.

The primary outcome of interest was major adverse cardiovascular events (MACE) composed of death, MI, stroke, or unplanned revascularization at 1-year follow-up. Secondary outcomes of interest included individual MACE components and clinical subgroups.

The collection and use of blood samples in the CAPITAL Biobank was approved by the Ottawa Health Science Network Research Ethics Board (Protocol #20160516-01H) and informed consent was obtained from all patients for collection. The outcomes study assessment was approved by Ottawa Health Science Network Research Ethics Board (#20190224-01H and #20180562-01H) to evaluate clinical outcomes following revascularization.

Statistical analysis

Data are reported as mean ± SD, median ± interquartile range, or number and percentage (%) where appropriate. Plasma adenosine levels were compared using Mann–Whitney *U* tests. Plasma adenosine levels were categorized into tertiles (33rd and 67th percentiles) and Kaplan–Meier plots generated to assess event distributions with comparison by log rank tests. Subsequent Cox proportional-hazard models were then used to determine hazard ratios (HRs) and 95% confidence intervals (CIs). Multivariable Cox

proportional-hazards model was also performed incorporating variables known to impact adenosine levels and post-revascularization MACE, including age (years), sex, diabetes mellitus type 2, hypertension, dyslipidemia, CAD, acute coronary syndrome, and revascularization, as documented previously and highlighted in univariate analysis (**Table S1**).[14,23] We estimated an incidence rate of 9.5% and 15.5% (effect size of 60%) between the lowest and highest adenosine tertiles with an $\alpha = 0.05$ and $\beta = 0.20$ which yielded a sample size of 476 per tertile for a total sample of 1,428 subjects. All statistical analyses were completed using SAS version 9.4 (SAS Institute, Cary, NC). All figures were created using GraphPad Prism version 8 (GraphPad Software, La Jolla, CA). The $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

During the study period, 1,815 patients underwent angiography and had blood collected in the CAPITAL Biobank for further analysis with 1-year follow-up completed. Within the collected samples, 1,323 had adenosine levels quantified from the time of index angiography and were included in our analysis. Of the 1,323 patients included, 675 (51.0%) underwent revascularization by either percutaneous coronary intervention (PCI; 70.8%) or coronary artery bypass grafting (CABG; 28.7%) or both (0.4%). Medical therapy was pursued in 648 patients (49.0%) with 64.5% having obstructive CAD and 35.5% have no obstructive CAD at the time of angiography (**Figure 1**). Mean age was 66.8 ± 11.6 years, 29.3% were women, risk factors included 65.2% with hypertension, 61.1% with dyslipidemia, 30.5% with diabetes, 18.1% active smokers, and 14.9% with a family history of CAD. Indications for angiography included 59.7% with acute coronary syndrome (ACS) and 37.7% with stable CAD. Patients had a history of PCI (27.7%), MI (23.3%), CABG (6.5%), peripheral artery disease (6.7%), and cerebrovascular accident (CVA; 6.5%). Medications at the time of angiography included aminosalicylic acid 90.9%), P2Y12 (89.4%), angiotensin-converting enzyme/angiotensin receptor blocker; 53.5%), beta-blocker (59.2%), and statin (80.9%; **Table 1**). Adenosine values were stratified according tertile (i) 0–33rd (low adenosine, $n = 436$) with a cutoff of 693.2 nM; (ii) 34–67th (middle adenosine, $n = 451$) with a cutoff level of 902.9 nM; and (iii) 68–100th (high adenosine, $n = 436$) with a cutoff level of 1141.9 nM with baseline characteristics stratified by tertiles (**Table 1**).

Patient outcomes

Patient outcomes were assessed at 1 year from their revascularization procedure, as previously described.[14] MACE occurred in 7.3% of patients at 1 year. When stratifying outcomes by adenosine tertiles we noted no significant differences in MACE rates between the tertiles (log rank $P = 0.83$). Similarly, no differences in HRs were noted when comparing the low to middle (HR 1.10, 95% CI 0.68–1.78, $P = 0.70$) or low to high adenosine tertiles (HR 0.95, 95% CI 0.56–1.57, $P = 0.84$; **Figure 2a**).

Secondary outcome analysis of the individual MACE components similarly revealed no changes among the subgroups (**Figure 2b–e**): death (log rank $P = 0.95$) and low vs middle (HR 1.02, 95% CI 0.55–1.92, $P = 0.94$) or low to high adenosine tertiles (HR 0.93, 95% CI 0.49–1.77, $P = 0.82$); MI (log rank $P = 0.95$), low vs. middle (HR 1.17, 95% CI 0.36–3.83, $P = 0.80$) or low to high adenosine tertiles (HR 0.98, 95% CI 0.28–3.38, $P = 0.98$); or unplanned revascularization (log rank $P = 0.49$); and low vs. middle (HR 1.01, 95% CI 0.53–1.90, $P = 0.98$) or low vs. high adenosine tertiles (HR 0.69, 95% CI 0.34–1.39, $P = 0.29$). When assessing CVA individually there was a noted difference with CVA stratified by adenosine tertiles (log rank $P = 0.03$), with no differences observed between low vs. middle (HR 4.37, 95% CI 0.95–20.23, $P = 0.059$) or low vs. high adenosine tertiles (HR 0.97, 95% CI 0.14–6.89, $P = 0.98$; **Figure 2c**). Adjusted analyses for age, ACS, diabetes mellitus type 2, sex, CAD, revascularization, hypertension, and dyslipidemia did not change the observed trends with no difference seen in MACE between low vs. middle (HR 1.11, 95% CI 0.68–1.80, $P = 0.68$) or low vs. high adenosine tertiles (HR 1.05, 95% CI 0.63–1.74, $P = 0.85$), nor in unplanned revascularization between low vs. middle (HR 1.06, 95% CI 0.56–2.01, $P = 0.86$) or low vs. high adenosine tertiles (HR 0.70, 95% CI, 0.34–1.42, $P = 0.32$; **Table 2**).

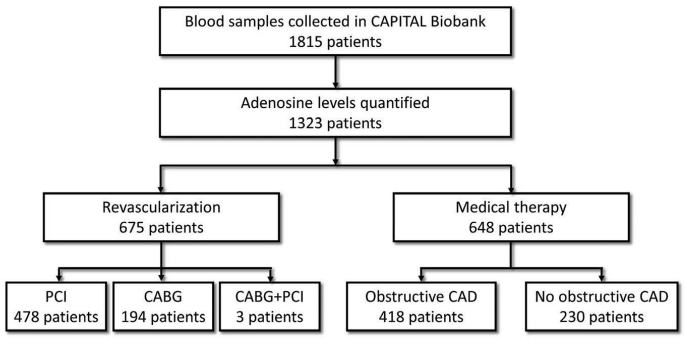


Figure 1 Patient flow diagram. CABG, coronary artery bypass grafting; CAD, coronary artery disease; CAPITAL, Cardiovascular and Percutaneous Intervention Trials; PCI, percutaneous coronary intervention.

Table 1 Baseline characteristics

	Total cohort		Adenosine tertiles					
			Low		Middle		High	
Number	1323		436		451		436	
Age, mean ± SD	66.8	11.6	67.0	11.4	67.3	11.8	66.2	11.6
Sex, female, *n* %	387	29.3	112	25.7	144	31.9	131	30.0
Hypertension, *n* %	863	65.2	287	65.8	281	62.3	295	67.7
Dyslipidemia, *n* %	808	61.1	266	61.0	277	61.4	265	60.8
Diabetes, *n* %								
Type I	8	0.6	4	0.9	1	0.2	3	0.7
Type II	396	29.9	132	30.3	116	25.7	148	33.9
Diet	24	1.8	8	1.8	7	1.6	9	2.1
Oral hypoglycemic agents	266	20.1	85	19.5	82	18.2	99	22.7
Insulin	106	8.0	39	8.9	27	6.0	40	9.2
Smoking, *n* %								
Never	765	57.8	261	59.9	253	56.1	251	57.6
Remote	319	24.1	95	21.8	113	25.1	111	25.5
Active	239	18.1	80	18.3	85	18.8	74	17.0
Family history of CAD, *n* %	197	14.9	71	16.3	56	12.4	70	16.1
Indication for angiography, *n* %								
ACS	790	59.7	176	40.4	176	39.0	181	41.5
Staged PCI	150	11.3	54	12.4	52	11.5	44	10.1
Stable CAD	499	37.7	174	39.9	173	38.4	152	34.9
Atrial fibrillation, *n* %	137	10.4	38	8.7	47	10.4	52	11.9
Medical history, *n* %								
PCI	366	27.7	124	28.4	119	26.4	123	28.2
MI	308	23.3	107	24.5	92	20.4	109	25.0
CABG	86	6.5	26	6.0	28	6.2	32	7.3
PAD	89	6.7	25	5.7	30	6.7	34	7.8
CVA	86	6.5	24	5.5	23	5.1	39	8.9
Bleed	23	1.7	9	2.1	4	0.9	10	2.3
Heart failure	100	7.6	33	7.6	25	5.5	42	9.6
Medications, *n* %								
ASA	1203	90.9	404	92.7	404	89.6	395	90.6
P2Y12	1183	89.4	401	92.0	395	87.6	387	88.8
ACEi/ARB	708	53.5	224	51.4	242	53.7	242	55.5
Beta-blocker	783	59.2	255	58.5	273	60.5	255	58.5
Calcium channel blocker	185	14.0	62	14.2	62	13.7	61	14.0
Statin	1070	80.9	364	83.5	360	79.8	346	79.4
Revascularized, *n* %	675	51.0	238	54.6	214	47.5	223	51.1

ACEi/ARB, angiotensin-converting enzyme inhibitor/angiotensin receptor blocker; ACS, acute coronary syndrome; ASA, acetylsalicylic acid; CABG, coronary artery bypass grafting; CAD, coronary artery disease; CVA, cardiovascular accident; MI, myocardial infarction; PAD, peripheral artery disease; PCI, percutaneous coronary intervention.

Subgroup analyses were performed to further assess three cohorts of patients—demonstrating no significant differences between adenosine tertiles in MACE or unplanned revascularization: (i) medical therapy for obstructive CAD (**Figure S1**)—MACE (log rank $P = 0.16$) and low vs. middle (HR 0.68, 95% CI 0.36–1.26, $P = 0.22$) or low vs. high adenosine tertiles (HR 0.54, 95% CI 0.27–1.06, $P = 0.07$), and unplanned revascularization (log rank $P = 0.77$) and low vs. middle (HR 1.02, 95% CI 0.41–2.58, $P = 0.96$) or low vs. high adenosine tertiles (HR 0.72, 95% CI 0.26–2.03, $P = 0.54$); (ii) revascularization cohort (**Figure S2**)—MACE (log rank $P = 0.16$) and low vs. middle (HR 1.89, 95% CI 0.75–4.74, $P = 0.17$) or low vs. high adenosine tertiles (HR 2.34, 95% CI 0.96–5.68, $P = 0.06$) and unplanned revascularization (log rank $P = 0.86$) and low vs. middle (HR 0.92, 95% CI 0.32–2.08, $P = 0.86$) or low vs. high adenosine tertiles (HR 0.79, 95% CI 0.31–2.00, $P = 0.62$); and (iii) ACS cohort (**Figure S3**)—MACE (log rank $P = 0.32$) and low vs. middle (HR 0.57, 95% CI 0.27–1.21, $P = 0.14$) or low vs. high adenosine tertiles (HR 0.74, 95% CI 0.37–1.50, $P = 0.41$)

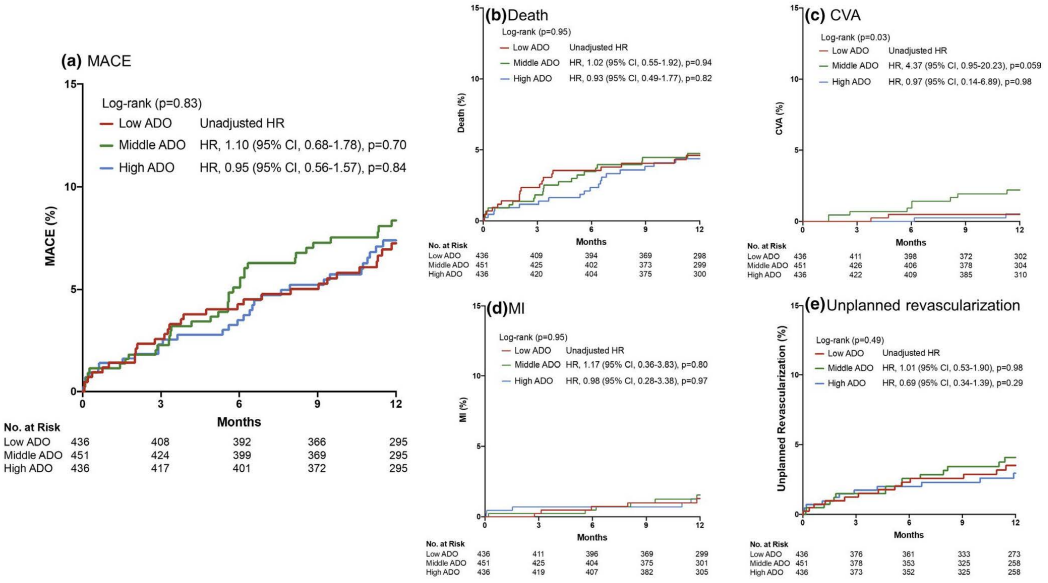


Figure 2 Adenosine levels and cardiovascular outcomes. (**a**) Major adverse cardiac events (MACE) including death, cerebrovascular accident (CVA), myocardial infarction (MI), and unplanned revascularization did not demonstrate any difference between middle or high adenosine tertiles. Similar trends were observed when analyzing by subgroups with no differences between tertiles noted for (**b**) death, (**d**) MI, and (**e**) unplanned revascularization. (**c**) CVA demonstrated higher incidence of stroke in the middle adenosine tertile. Adenosine levels stratified into tertiles (0–33rd, 34–66th, and 67–100th) for analysis. Kaplan–Meier curves generated and compared via log rank with subsequent unadjusted hazard ratios (HRs) compared by Cox proportional hazards model. $P < 0.05$ was defined as statistically significant. ADO, adenosine levels; CI, confidence interval.

and unplanned revascularization (log rank $P = 0.96$) and low vs. middle (HR 1.12, 95% CI 0.45–2.75, $P = 0.81$) or low vs. high adenosine tertiles (HR 1.01, 95% CI 0.40–2.55, $P = 0.98$). Moreover, from a clinical perspective, those who experienced MACE were more likely to be older (72.4 ± 11.5 vs. 66.1 ± 11.4 years, $P < 0.0001$) and have hypertension (73.4% vs. 64.1%, $P = 0.02$), dyslipidemia (72.2% vs. 59.6%, $P = 0.002$), diabetes (46.2% vs 27.7%, $P < 0.0001$), and a history of MI (30.4% vs. 22.3%, $P = 0.02$), CABG (10.8% vs. 5.9%, $P = 0.02$), and peripheral artery disease (11.4% vs. 6.1%, $P = 0.01$). Patients with MACE were less likely to present as stable CAD (29.1% vs. 38.9%, $P = 0.02$) and less likely to undergo revascularization (39.2% vs. 52.6%, $P = 0.002$; **Table S1**).

Finally, we evaluated adenosine values within each outcome cohort with no significant differences noted in any comparison (**Figure 3**): (i) MACE—absent (n = 1,227) vs. present (n = 96, 902.2 nM (621.4–1,292.3 nM) vs. 914.2 585.0–1,324.5 nM), $P = 0.86$); (ii) death—absent (n = 1,266) vs. present (n = 57, 904.1 nM (621.4–1,285.2 nM] vs. 857.0 nM (540.8–1,418.7 nM), $P = 0.68$); (iii) MI—absent (n = 1307) vs. present (n = 16, 902.8 nM (619.9–1,292.3 nM) vs. 838.7 nM (562.8–1,294.9 nM), $P = 0.77$); (iv) CVA—absent (n = 1,310) vs. present (n = 13; 902.2 nM (619.1–1,295.7 nM)

Table 2 Clinical outcomes predicted by plasma adenosine levels

	MACE			Unplanned revascularization		
	Event rates (%)	HR (95% CI)	*P* value	Event rates (%)	HR (95% CI)	*P* value
Low adenosine	7.1	–		4.4	–	
Unadjusted analysis (relative to low adenosine)						
Middle adenosine	7.8	1.10 (0.68–1.78)	0.70	4.2	1.01 (0.53–1.90)	0.98
High adenosine	6.9	0.95 (0.56–1.57)	0.84	3.0	0.69 (0.34–1.39)	0.29
Adjusted analysis (relative to low adenosine)[a]						
Middle adenosine	7.8	1.11 (0.68–1.80)	0.68	4.2	1.06 (0.56–2.01)	0.86
High adenosine	6.9	1.05 (0.63–1.74)	0.85	3.0	0.70 (0.34–1.42)	0.32

CI, confidence interval; HR, hazard ratio; MACE, major adverse cardiac event.
[a]Adjusted for age (years), sex, type 2 diabetes, hypertension, dyslipidemia, coronary artery disease, acute coronary syndrome, and revascularization.

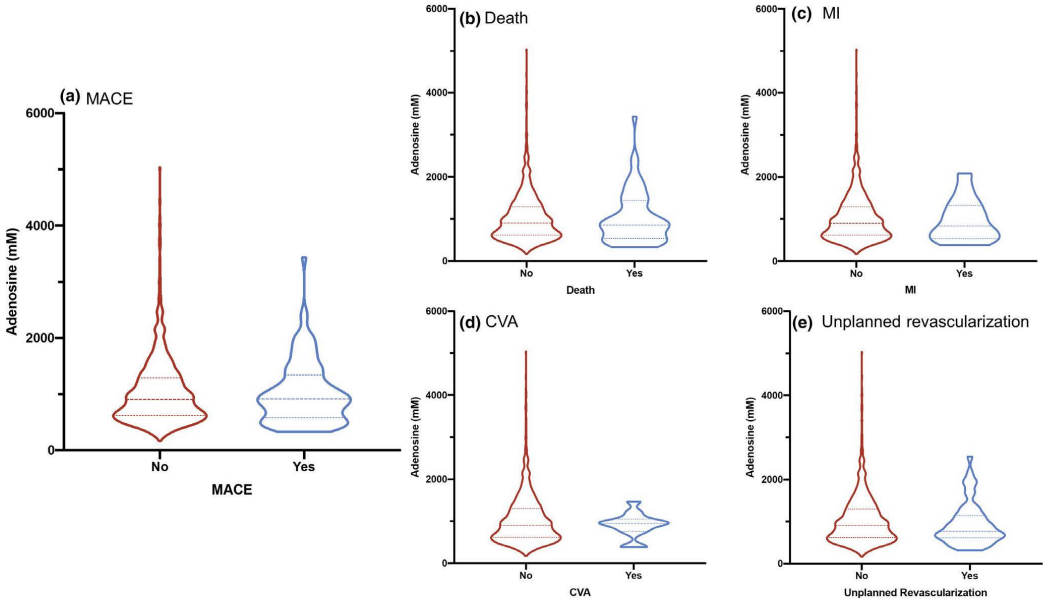


Figure 3 Comparison of adenosine levels across cardiovascular outcome groups. Circulating adenosine values presented according to each cohort with no significant differences noted among: (**a**) MACE—absent (n = 1,165) vs. present (n = 158, 902.8 nM (619.9–1,285.2 nM) vs. 904.0 (616.7–1,295.7 nM), P = 0.99). (**b**) Death—absent (n = 1,255) vs. present (n = 68, 903.3 nM (621.4–1,283.1 nM) vs. 897.8 nM (543.9–1,458.1 nM), P = 0.99). (**c**) MI—absent (n = 1,293) vs. present (n = 30, 902.8 nM (619.9–1,292.3 nM) vs. 907.0 nM (709.2–1,341.0 nM), P = 0.63). (**d**) CVA—absent (n = 1,294) vs. present (n = 29, 897.6 nM (618.3–1,292.3 nM] vs. 970.0 nM (837.0–1,256.7 nM), P = 0.25). (**e**) Unplanned revascularization—absent (n = 1,263) vs. present (n = 60, 905.7 nM (619.6–1,302.2 nM) vs. 789.8 nM (616.0–1,127.2), P = 0.15). Data was presented as median (interquartile range (IQR)) and compared using Mann–Whitney U test. $P < 0.05$ was defined as statistically significant. CVA, cerebrovascular accident; MACE, major adverse cardiac events; MI, myocardial infarction.

vs. 953.2 nM (802.0–1,007.3 nM), P = 0.82); (v) unplanned revascularization—absent (n = 1,272) vs. present (n = 51, 904.3 nM (619.9–1,297.9 nM) vs. 770.7 nM (615.4–1,144.8), P = 0.26).

DISCUSSION

In the current study, we sought to evaluate the ability of plasma adenosine levels to risk stratify patients and predict MACE. Contrary to our hypothesis, adenosine levels were not found to predict MACE rates at 1 year of follow-up, nor did it predict any of the individual components. Multivariable adjustment for factors known to influence outcomes and adenosine levels similarly did not demonstrate a clear role of adenosine for risk stratifying patients at the time of invasive angiography. Hence, this study does not support the use of plasma adenosine in risk stratification of cardiac patients for 1 year events.

Prior to our analysis, adenosine's role as a predictor of CV outcomes was inconclusive. Individuals with genetic mutations (AMPD1) leading to reduced adenosine deaminase activity provide a unique perspective on the role of adenosine in CV outcomes. Elevated adenosine levels have been noted in patients with heart failure (HF) previously, supporting a compensatory role as a counter-regulatory molecule in response to rising catecholamine levels endemic to patients with HF.[24] Therapeutically, dipyridamole-induced adenosine augmentation was suggested to improve HF severity.[25] Moreover, less-adverse outcomes have been noted in patients with HF with AMPD1 mutations; although adenosine levels were not assessed, limiting discernment as to whether circulating or tissue adenosine differences led to the observed changes.[26,27] Indeed, AMPD1 is most active in the skeletal muscle, suggesting tissue levels may be more directly affected.[28] Moreover, when 367 patients with obstructive CAD were stratified by AMPD1 status, they demonstrated improved CV outcomes, although adenosine levels were not quantified.[15] However, 161 patients undergoing revascularization (PCI and CABG) did not demonstrate differential circulating adenosine levels by AMPD1 status, nor did AMPD1 status predict CV outcomes, suggesting alternative pathways for its observed effects.[16] Indeed, these discrepant results reflect an incomplete understanding of adenosine biology, in that the observed outcome effects may actually be driven by modification of local cardiac/vascular adenosine levels with systemic adenosine levels providing a relatively minor role as supported by work in patients with HF.[16,29] Similarly, our findings may suggest that local adenosine levels may be more relevant and predictive of vascular healing. While

direct coronary sampling has demonstrated reduced local adenosine levels post-PCI,[30] the technical barriers of this approach would preclude meaningful adoption as a clinical tool.

Whereas circulating adenosine levels may not predict future events, this does not exclude adenosine as a viable therapeutic target, particularly when considering its tissue-level characteristics. As discussed, numerous agents known to modulate adenosine have shown favorable impacts on vascular healing and cardiovascular outcomes, including dipyridamole,[1] cilostazol,[7,8] and ticagrelor.[9] Although all of these agents have demonstrated favorable impact on clinical outcomes, the mechanism of action may be on local rather than circulating adenosine levels. Dipyridamole is commonly used in i.v. formulation leading to transient peaks in central adenosine levels[31] lessening with repeated stimuli,[32] whereas sustained oral administration up to 5 days demonstrated early changes in circulating adenosine, lessening after 48 hours.[33] However, dipyridamole is known to more potently inhibit ENT-1 than ticagrelor, prolonging detectable levels of adenosine following adenosine administration in the presence of both agents.[34] Although ticagrelor has been shown to augment circulating adenosine in the setting of ACS, other studies have failed to consistently demonstrate this effect.[12,13] Taken together, these findings suggest that although therapeutic modulation of adenosine may represent a viable target, the effect on adenosine levels may occur transiently at a systemic level, with more sustained augmentation locally. Moreover, adenosine receptor levels have been shown to dynamically respond to changes in adenosine levels, reflecting yet another pathway by which vasculoprotective effects are mediated, while not appreciably impacting systemic adenosine levels.[35] Indeed, using systemic adenosine levels as a target diagnostically or therapeutically is challenged by its innate properties, variations between local and systemic levels and the complex homeostatic mechanisms regulating adenosine levels. Hence, although monitoring adenosine levels may not provide prognostication abilities, modulating adenosine biology still carries significant promise as a therapeutic approach and remains the focus of ongoing investigation.

Our study is not without limitations. First, our population is heterogenous with a variety of indications for invasive angiography and differential revascularization strategies, although assessment of these various subgroups similarly failed to demonstrate prognostic effects. Second, our follow-up period was limited to 1 year and we cannot rule out the possibility that adenosine may predict longer term outcomes; however, we are unable to determine this within the confines of the current study. Finally, systemic quantification of adenosine levels is subject to biological and analytical variation, which may impact levels quantified.[14]

CONCLUSION

Systemic adenosine levels are not predictive of CV events at one year in patients undergoing angiography. Although therapeutic targeting of adenosine and associated pathways may yet be beneficial, risk stratification using this molecule is not clinically useful.

Supporting Information. Supplementary information accompanies this paper on the *Clinical and Translational Science* website (www.cts-journal.com).

Funding. This work was supported by the UOHIAMO AFP Innovations Funding Competition for Innovative Clinical Projects and CFI (Canadian Foundation for Innovation). The Vered-Beanlands Endowed Fellowship (T.S.). The Canadian Institutes of Health Research (Vanier Research Graduate Scholarship (R.G.J.) and Banting Postdoctoral Fellowship (F.D.R.)), the Royal College of Physicians and Surgeons of Canada (Detweiler Travelling Fellowship (F.D.R.)).

Conflicts of Interest. The authors declared no competing interests for this work.

Author Contributions. T.S., R.G.J., and B.H. designed the research and wrote the manuscript. T.S., R.G.J., A.L., C.G., P.M., S.P., J.J., R.M., J.M., P.B., S.P., J.J.R., A.Y.C., D.S., M.F., M.L.M., and B.H. performed the research. T.S., R.G.J., F.D.R., and P.D.S. analyzed the data. T.S. and C.G. contributed new reagents/analytical tools.

1. Simard, T. *et al.* Adenosine as a marker and mediator of cardiovascular homeostasis: a translational perspective. *Cardiovasc. Hematol. Disord. Drug Targets* **19**, 109–131 (2018).
2. Loffler, M., Morote-Garcia, J.C., Eltzschig, S.A., Coe, I.R. & Eltzschig, H.K. Physiological roles of vascular nucleoside transporters. *Arterioscler. Thromb. Vasc. Biol.* **27**, 1004–1013 (2007).
3. Yegutkin, G.G. Nucleotide- and nucleoside-converting ectoenzymes: important modulators of purinergic signalling cascade. *Biochim. Biophys. Acta* **1783**, 673–694 (2008).
4. Chen, J.-F., Eltzschig, H.K. & Fredholm, B.B. Adenosine receptors as drug targets—what are the challenges? *Nat. Rev. Drug Discov.* **12**, 265–286 (2013).
5. Kim, H.-H. & Liao, J.K. Translational therapeutics of dipyridamole. *Arterioscler. Thromb. Vasc. Biol.* **28**, s39–s42 (2008).
6. Al Jaroudi, W. & Iskandrian, A.E. Regadenoson: a new myocardial stress agent. *J. Am. Coll. Cardiol.* **54**, 1123–1130 (2009).
7. Liu, Y. *et al.* Inhibition of adenosine uptake and augmentation of ischemia-induced increase of interstitial adenosine by cilostazol, an agent to treat intermittent claudication. *J. Cardiovasc. Pharmacol.* **36**, 351–360 (2000).
8. Douglas, J.S. *et al.* Coronary stent restenosis in patients treated with cilostazol. *Circulation* **112**, 2826–2832 (2005).
9. Nylander, S. & Schulz, R. Effects of P2Y12 receptor antagonists beyond platelet inhibition–comparison of ticagrelor with thienopyridines. *Br. J. Pharmacol.* **173**, 1163–1178 (2016).
10. Van Giezen, J.J.J., Sidaway, J., Glaves, P., Kirk, I. & Björkman, J.A. Ticagrelor inhibits adenosine uptake in vitro and enhances adenosine-mediated hyperemia responses in a canine model. *J. Cardiovasc. Pharmacol. Ther.* **17**, 164–172 (2012).
11. Li, X., Wang, Q., Xue, Y., Chen, J. & Lv, Q. Ticagrelor compared with clopidogrel increased adenosine and cyclic adenosine monophosphate plasma concentration in acute coronary syndrome patients. *Basic Clin. Pharmacol. Toxicol.* **120**, 610–614 (2017).
12. Ariotti, S. *et al.* Effects of ticagrelor, prasugrel, or clopidogrel on endothelial function and other vascular biomarkers: a randomized crossover study. *JACC Cardiovasc. Interv.* **11**, 1576–1586 (2018).
13. Bonello, L. *et al.* Ticagrelor increases adenosine plasma concentration in patients with an acute coronary syndrome. *J. Am. Coll. Cardiol.* **63**, 872–877 (2014).
14. Simard, T. *et al.* Evaluation of plasma adenosine as a marker of cardiovascular risk: analytical and biological considerations. *J. Am. Heart Assoc.* **8**, e012228 (2019).
15. Anderson, J.L. *et al.* A common variant of the AMPD1 gene predicts improved cardiovascular survival in patients with coronary artery disease. *J. Am. Coll. Cardiol.* **36**, 1248–1252 (2000).
16. Andreassi, M.G. *et al.* AMPD1 (C34T) polymorphism and clinical outcomes in patients undergoing myocardial revascularization. *Int. J. Cardiol.* **101**, 191–195 (2005).
17. Madhavan, M.V. *et al.* Stent-related adverse events >1 year after percutaneous coronary intervention. *J. Am. Coll. Cardiol.* **75**, 590–604 (2020).
18. Albayrak, G. *et al.* Inhibitory effect of adenosine on intimal hyperplasia and proliferation of smooth muscle cells in a carotid arterial anastomosis animal model. *Vascular* **23**, 124–131 (2015).

19. Huszar, E.B., Barát, E. & Kollai, M. Isocratic high-performance liquid chromatographic determination of plasma adenosine. *Chromatographia* **42**, 318–322 (1996).
20. Simard, T. *et al.* Impact of center experience on patient radiation exposure during transradial coronary angiography and percutaneous intervention: a patient-level, international, collaborative, multi-center analysis. *J. Am. Heart Assoc.* **5**, e003333 (2016).
21. Le May, M.R. *et al.* A citywide protocol for primary PCI in ST-segment elevation myocardial infarction. *N. Engl. J. Med.* **358**, 231–240 (2008).
22. Thygesen, K. *et al.* Fourth universal definition of myocardial infarction (2018). *Circulation* **138**, e618–e651 (2018).
23. Jung, R.G. *et al.* Evaluation of plasminogen activator inhibitor-1 as a biomarker of unplanned revascularization and major adverse cardiac events in coronary angiography and percutaneous coronary intervention. *Thromb. Res.* **191**, 125–133 (2020).
24. Funaya, H. *et al.* Plasma adenosine levels increase in patients with chronic heart failure. *Circulation* **95**, 1363–1365 (1997).
25. Kitakaze, M. *et al.* Elevation of plasma adenosine levels may attenuate the severity of chronic heart failure. *Cardiovasc. Drugs Ther.* **12**, 307–309 (1998).
26. Feldman, A.M., Wagner, D.R. & McNamara, D.M. AMPD1 gene mutation in congestive heart failure. *New Insights Pathobiol. Dis. Progr.* **99**, 1397–1399 (1999).
27. Loh, E. *et al.* Common variant in AMPD1 gene predicts improved clinical outcome in patients with heart failure. *Circulation* **99**, 1422–1425 (1999).
28. Morisaki, T. *et al.* Molecular basis of AMP deaminase deficiency in skeletal muscle. *Proc. Natl. Acad. Sci.* **89**, 6457–6461 (1992).
29. Kalsi, K. Decreased cardiac activity of AMP deaminase in subjects with the AMPD1 mutation—a potential mechanism of protection in heart failure. *Cardiovasc. Res.* **59**, 678–684 (2003).
30. Guieu, R. *et al.* The use of HPLC to evaluate the variations of blood coronary adenosine levels during percutaneous transluminal angioplasty. *Clin. Chim. Acta* **230**, 63–68 (1994).
31. Hegedus, K., Keresztes, T., Fekete, I. & Molnar, L. Effect of i.v. dipyridamole on cerebral blood flow, blood pressure, plasma adenosine and cAMP levels in rabbits. *J. Neurol. Sci.* **148**, 153–161 (1997).
32. Pasini, F.L. *et al.* Pharmacological preconditioning of ischemic heart disease by low-dose dipyridamole. *Int. J. Cardiol.* **56**, 17–27 (1996).
33. German, D.C., Kredich, N.M. & Bjornsson, T.D. Oral dipyridamole increases plasma adenosine levels in human beings. *Clin. Pharmacol. Ther.* **45**, 80–84 (1989).
34. Nylander, S. *et al.* Ticagrelor inhibits human platelet aggregation via adenosine in addition to P2Y12 antagonism. *J. Thromb. Haemost.* **11**, 1867–1876 (2013).
35. Fenouillet, E. *et al.* Adenosine receptor profiling reveals an association between the presence of spare receptors and cardiovascular disorders. *Int. J. Mol. Sci.* **20**, 5964 (2019).

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Appendix J

Clinical features, sex differences and outcomes of myocardial infarction with nonobstructive coronary arteries: a registry analysis

Citation information:

Jung RG, Parlow S, Simard T, Chen C, Ghataura H, Kishore A, Parera A, Moreland R, Hughes I, Tavella R, Hibbert B, Beltrame J, Singh K. Clinical features, sex differences and outcomes of myocardial infarction with nonobstructive coronary arteries (MINOCA): a multi-centre, international, registry analysis. **(2020)**. *Coronary Artery Disease.*

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Author: Richard Jung, Simon Parlow, Trevor Simard, et al

Publication: Coronary Artery Disease

Publisher: Wolters Kluwer Health, Inc.

Date: May 3, 2020

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Clinical features, sex differences and outcomes of myocardial infarction with nonobstructive coronary arteries: a registry analysis

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Introduction **Clinical characteristics and outcomes of patients diagnosed with myocardial infarction (MI) with nonobstructive coronary arteries (MINOCA) remain largely unknown. Furthermore, we do not yet understand if women with MINOCA have worse outcomes similar to what has historically been observed with MI. The aims of the current study were to evaluate the (1) incidence of MINOCA in patients presenting with MI, (2) compare in-hospital outcomes of MINOCA and obstructive atherosclerotic coronary artery disease MI (OACD-MI), and (3) comparison of in-hospital clinical outcomes of patients with MINOCA stratified by sex.**

Methods and results **In this observational study, we combined data from two large university hospitals from Canada and Australia. Clinical characteristics and in-hospital outcomes of MINOCA and OACD-MI were analyzed by matching these patients in a 1:1 ratio after selecting patients with OACD-MI by systematic random sampling. Clinical characteristics associated with MINOCA were identified through multivariate logistic regression. Primary outcome of interest was net adverse cardiovascular events (NACE) defined as death, heart failure, stroke, and major bleeding. The incidence rate of MINOCA was 9.5%. Women, absence of traditional cardiac risk factors, and absence of ST-deviations on ECG were associated with diagnosis of MINOCA on angiography. NACE (P=0.0001), death (P=0.019), stroke (P=0.002), and heart failure (P=0.001) were significantly lower in patients with MINOCA. Subgroup analysis of women and men diagnosed with MINOCA revealed similar in-hospital outcomes.**

Conclusion **The incidence of MINOCA was 9.5%. Compared to OACD-MI, patients with MINOCA have less cardiac risk factors. In-hospital outcomes of patients diagnosed with MINOCA were better than OACD-MI.**
Coron Artery Dis **XXX: 000–000 Copyright © 2020 Wolters Kluwer Health, Inc. All rights reserved.**

Coronary Artery Disease 2020, **XXX**:000–000

Keywords: myocardial infarction with nonobstructive coronary arteries, sex differences, net adverse cardiovascular events

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Received 12 February 2020 Accepted 26 March 2020

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Introduction

Myocardial infarction (MI) is a cardiac condition that requires immediate diagnosis and treatment to reduce mortality and morbidity [1]. Coronary plaque rupture related thrombotic occlusion is the most common underlying pathology for MI [2,3]. The utilization of coronary angiography for management of patients presenting with MI has grown dramatically in the past two decades. Routine use of coronary angiography has revealed that epicardial coronary artery thrombotic occlusion is not the only cause of MI [4]. Nonthrombotic coronary etiologies such as epicardial coronary vasospasm, microvascular dysfunction, and myocardial pathologies such as Takotsubo cardiomyopathy can also present as MI. After ruling out the traditional and nontraditional causes of MI, we are still left with a significant proportion of patients presenting with MI where the cause remains unknown. The term 'Myocardial infarction with nonobstructive coronary arteries (MINOCA)' is coined for such diagnosis which is clinically defined by the presence of the universal acute MI criteria, absence of obstructive coronary artery disease (≥50% stenosis), and no overt cause for the clinical presentation at the time of coronary angiography [5]. At this time, patient-level data on clinical characteristics and outcomes of patients diagnosed with MINOCA remains unknown. Nevertheless, from early studies and systematic reviews we now know that unlike traditional MI, women constitute a large proportion of the MINOCA population. However, we do not know

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DOI: 10.1097/MCA.0000000000000903

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if women tend to have a worse outcome with MINOCA similar to MI, and if the underlying clinical characteristics vary according to the sex in the MINOCA group.

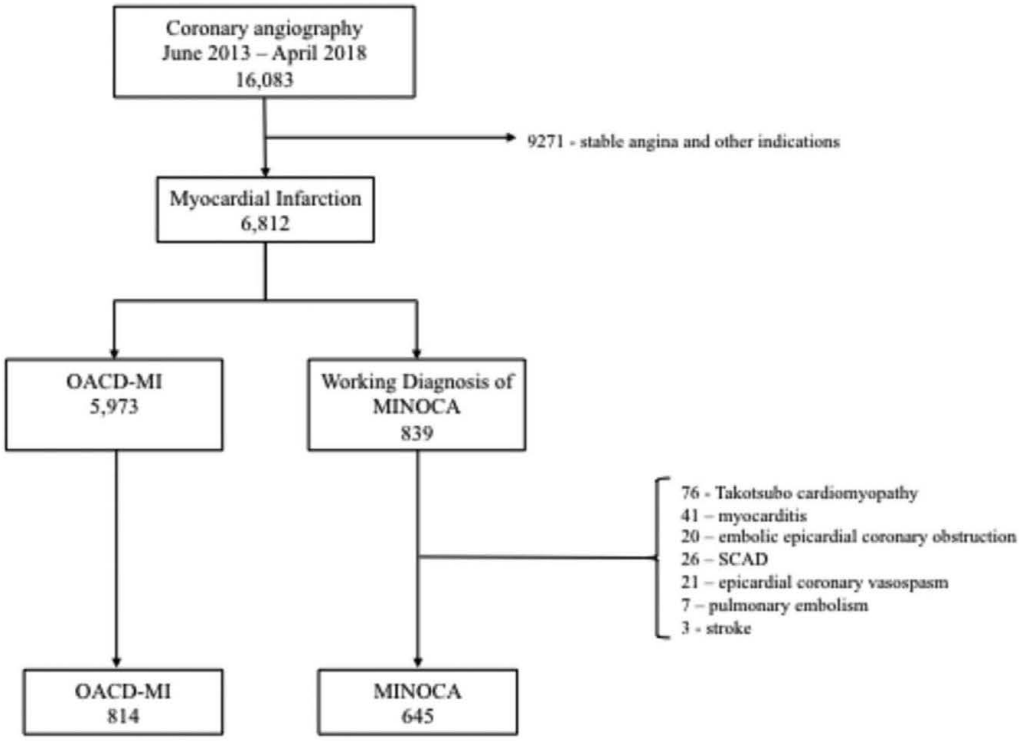
In the current study, we combined data from two large teaching hospitals from Canada and Australia with the aim to find:(1) the incidence of MINOCA in a large subset of patients presenting with MI, (2) the comparison of clinical characteristics and net adverse cardiovascular events (NACE) between the patients presenting with MINOCA and patients presenting with atherosclerotic coronary occlusion, and (3) the differences in clinical characteristics and NACE outcomes among patients with MINOCA by sex of the patient.

Methods

Study population and procedure

We performed an analysis of consecutive patients who were admitted with a diagnosis of MI at the University of Ottawa Heart Institute, Canada and at the Gold Coast University Hospital, Australia. The University of Ottawa Heart Institute is a high volume, tertiary care revascularization center [6]. Patients undergoing coronary angiography and revascularization at the University of Ottawa Heart Institute are included in the Cardiovascular And Percutaneous CliInical TriALs (CAPITAL) revascularization registry (Protocol No. 20140411-01H). From 15 October 2015 to 20 April 2018, a total of 10472 cases were prospectively collected. Similarly, Gold Coast University Hospital is a high volume tertiary care revascularisation center and is a part of the Queensland Cardiac Outcomes Registry where clinical data is prospectively collected from various sub-specialties such as acute coronary syndrome (ACS; interventional cardiology), cardiac and thoracic surgery, heart failure, cardiac imaging/echocardiography, electrophysiology and pacing, and cardiac rehabilitation. From June 2013 to 31 December 2016, a total of 5611 cases were prospectively collected from the Gold Coast University Hospital (HREC/17/QGC/174), resulting in a total of 16083 cases (Fig. 1). Both prospective

Fig. 1



Flowchart showing final diagnosis of patients who underwent coronary angiography.

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registries had the same definition for clinical characteristics and outcomes. Patients were prospectively enrolled into these registries if they presented with an indication for angiography and were prospectively recorded as an ACS, defined as ST-elevation myocardial infarction (STEMI), non-ST-elevation myocardial infarction (NSTEMI) or unstable angina were extracted for further analysis. Of these cases, those with nonobstructive coronary artery disease on index angiography, defined as stenosis <50% were then selected for detailed chart review. In the cohort of patients with nonobstructive coronary disease, we diagnosed MINOCA using the European Society of Cardiology diagnostic criteria for MINOCA [5]. These selected cases then underwent a complete chart review to confirm the diagnosis of MINOCA and then subsequent data collection as noted when available. We excluded patients with MI who were managed noninvasively and did not undergo coronary angiography. Furthermore, after careful examination of angiographic findings or with other noninvasive imaging techniques, patients who were diagnosed with spontaneous coronary artery dissection, Takotsubo cardiomyopathy, myocarditis, epicardial coronary artery spasm, or coronary artery embolism were excluded. Patients were excluded if they were diagnosed with type II MI and noncardiac causes of a significant rise in troponin such as a large pulmonary embolus or intracranial event. In the end, MINOCA group comprised of patients who had an absence of >50% coronary disease and no obvious epicardial coronary, noncoronary or myocardial cause of rise in the troponin with or without significant ECG changes. In order to compare the clinical characteristics and hospital outcomes between MINOCA and MI, data from patients with confirmed diagnosis of obstructive atherosclerotic coronary artery disease (OACD-MI) was also obtained.

Data collection outcome measures

Individual patient diagnosis, demographic data, risk factors, and outcome data were collected prospectively using the Queensland Cardiovascular Outcomes Registry for the Gold Coast University Hospital and using the CAPITAL revascularization registry at the University of Ottawa Heart Institute. Information which were not available in the database such as blood test results, noninvasive cardiac and noncardiac imaging results, and medication history were obtained using electronic medical records. The primary outcome of interest was in-hospital net adverse cardiac events, defined as a composite of all-cause mortality, new diagnosis of heart failure, stroke, and major bleeding. The secondary outcomes of interest were in-hospital rate of all-cause mortality, new diagnosis of heart failure, stroke, and major bleeding.

Definitions

MI was defined by the well-established 'Third Universal Definition of Myocardial Infarction' [4]. This contemporary definition focuses on a combination of positive troponin (>99th percentile) with a change in troponin levels upon serial measurement and corroborative clinical evidence of an acute MI [7]. STEMI was defined as the presence of at least 1 mm ST-segment elevation in ≥2 contiguous pericardial leads on a standard 12- lead ECG. NSTEMI was defined as serial rise in the troponin levels as defined above with or without ischemic changes on the ECG. Angiographic classification for MINOCA required absence of significant coronary artery disease, which was defined as less than 50% narrowing in all the coronary arteries on coronary angiography. New diagnosis of heart failure was defined as a reduction in LVEF ≤40% and typical symptoms (e.g. breathlessness, ankle swelling, and fatigue) [8]. Stroke was defined as an acute episode of focal or global neurological dysfunction caused by brain, spinal cord, or retinal vascular injury as a result of hemorrhage or infarction.

Statistical analysis

Baseline characteristics including age, sex, past medical history, ECG findings, medication at admission and discharge, investigations, and in-hospital outcomes were compared between patients with MINOCA and those with MI because of OACD-MI. The MI patients with OACD-MI for comparison were selected by systematic random sampling from the whole cohort of patients with MI. The sampling interval was calculated by dividing the population size by the desired sample size. Comparison of patients presenting with MINOCA and MI with obstructive coronary disease were done in a 1:1 ratio; for example, we expected that roughly 800 patients with MINOCA would be identified (as expected from previously published data) [9], then 800 patients with MI will be randomly selected by systematic random sampling for comparison. Because there has been no change in the incidence, clinical characteristics, diagnosis and management of the MI during the time interval selected, sample of 800 patients with MI will represent complete MI cohort without any significant bias. We then compared the baseline characteristics between women diagnosed with MINOCA and the OACD-MI. Further comparisons were then made between men and women diagnosed with MINOCA.

Continuous variables were summarized as mean and SD or median and interquartile range and compared between the groups using the Student's *t*-test and the Mann–Whitney *U*-test, respectively. Categorical variables were summarized as percentages and compared between the groups applying the Pearson χ^2 test or Fisher's exact test as appropriate. Multivariate logistic regression was performed for clinical variables that were significant in the univariate analysis to identify clinical characteristics that were independently associated with MINOCA diagnosis. A P-value <0.05 was considered significant. Statistical analysis was performed using SAS v9.4 (SAS Institute Inc., Cary, North Carolina, USA).

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Results

Incidence

A total of 16083 patients underwent coronary angiography of which MI was an indication in 6812 patients. On coronary angiography, 839 (12%) of the patients did not have OACD-MI. This subgroup of patients constituted of embolic epicardial coronary obstruction (2.3%), spontaneous coronary obstruction (3.1%), epicardial coronary vasospasm (2.5%), myocarditis (4.9%), Takotsubo cardiomyopathy (9.0%), noncardiac causes (1.2%), and MINOCA (76.9%) (Fig. 1). The overall incidence of MINOCA in those initially presenting with MI was 9.5%.

Myocardial infarction with nonobstructive coronary arteries and obstructive atherosclerotic coronary artery disease myocardial infarction

There was no difference in the average age between the two groups (Table 1). A significantly higher proportion of women had the diagnosis of MINOCA compared to OACD-MI (46 vs. 25%, $P=0.0001$). Traditional cardiovascular risk factors and cardiovascular disease were more prevalent among patients diagnosed with OACD-MI. Only 16% of the patients with MINOCA had ischemic ECG changes (either ST-elevation or depression) compared to 64% of patients presenting with OACD-MI. There was a significant difference in the medications commenced at admission and at discharge between the two groups. Patients with MINOCA had lower level of low-density lipoprotein (LDL) cholesterol, hemoglobin, and plasma creatinine levels. Both groups had high prevalence of atrial fibrillation without any difference. The combined endpoint of NACE (2.9 vs. 8.9%, $P=0.00003$) was reduced in the MINOCA group. Death (0.3 vs. 1.8%, $P=0.019$), stroke (0.16 vs. 2.3%, $P=0.002$), and heart failure (2.6 vs. 6.8%, $P=0.001$) was significantly lower in patients with MINOCA. Similar results were noted in clinical characteristics when comparison was made between women diagnosed with MINOCA and OACD-MI (Table 2).

Table 1 **Clinical characteristics and in-hospital outcomes**

	MINOCA (645) (%)	OACD-MI (814) (%)	*P* value
Age (years) (mean±SD)	62.12±12	61.12±12	0.94
Gender (female)	299 (46%)	206 (25%)	0.0001
History			
Hypertension	361 (56%)	486 (61%)	0.045
Diabetes mellitus	119 (18%)	196 (24%)	0.018
Congestive heart failure	14 (2.2%)	59 (7.5%)	0.0001
Myocardial infarction	26 (9%)	195 (25%)	0.0001
Stroke	40 (6.2%)	52 (6.6%)	0.64
Cancer	18 (2.8%)	55 (6.9%)	0.001
Dementia	3 (0.5%)	11 (1.4%)	0.08
COPD	45 (7%)	64 (8%)	0.49
Peripheral vascular disease	11 (1.7%)	37 (4.6%)	0.013
Atrial fibrillation	63 (10%)	79 (10%)	0.66
Smoking	139 (22%)	340 (42%)	0.001
ECG changes			
ST elevation	60 (10%)	321 (40%)	0.001
ST depression	40 (6.6%)	191 (24%)	0.001
Admission medications			
Aspirin	439 (68%)	616 (78%)	0.0001
Warfarin	34 (5.2%)	17 (2.7%)	0.002
B-blocker	294 (46%)	409 (52%)	0.012
ARB	98 (15%)	151 (19%)	0.046
ACE inhibitor	222 (35%)	277 (35%)	0.77
Statin	434 (67%)	520 (66%)	0.68
Discharge medications			
Aspirin	209 (42%)	707 (91%)	0.0001
B-blocker	159 (32%)	544 (69%)	0.0001
ARB	129 (26%)	120 (15%)	0.0001
ACE inhibitor	119 (24%)	381 (48%)	0.0001
Statin	264 (54%)	715 (90%)	0.0001
Blood results			
Creatinine (mean±SD), µmol/L	87±75	102±85	0.0004
LDL cholesterol (mean±SD), (mmol/L)	2.06±1.1	2.95±1.2	0.0001
Hemoglobin (mean±SD), g/dL	132±28	139±19	0.0001
Platelets (mean±SD), $\times 10^9$ per L	232±83	227±67	0.86
Outcomes			
NACE	19 (2.9%)	72 (8.9%)	0.0001
Heart failure	17 (2.6%)	55 (6.8%)	0.001
Death	2 (0.3%)	15 (2%)	0.019
Stroke	1 (0.16%)	18 (2.3%)	0.002
Major bleeding	3 (0.45%)	24 (3%)	0.02

ARB, Angiotensin II receptor blocker, COPD, Chronic obstructive pulmonary disease; LDL, low-density lipoprotein; MINOCA, myocardial infarction with nonobstructive coronary arteries; NACE, net adverse cardiovascular events; OACD-MI, obstructive atherosclerotic coronary artery disease myocardial infarction.

Table 2 **Difference in clinical characteristics and outcomes among women diagnosed with myocardial infarction with nonobstructive coronary arteries and obstructive atherosclerotic coronary artery disease myocardial infarction**

	Women		
	MINOCA (*n*=299)	OACD-MI (*n*=206)	*P* value
Age (years) (mean±SD)	64.4±12	63.4±12	0.81
Gender (female)	299 (46%)	206 (25%)	0.0001
History			
Hypertension	166 (55%)	123 (62%)	0.16
Diabetes mellitus	51 (17%)	45 (22%)	0.15
Congestive heart failure	8 (2.7%)	19 (9.6%)	0.001
Myocardial infarction	7 (5%)	29 (15%)	0.015
Stroke	17 (5.7%)	15 (7.5%)	0.41
Cancer	11 (3.7%)	16 (7.8%)	0.04
Dementia	1 (0.3%)	3 (1.5%)	0.16
COPD	27 (9%)	16 (8%)	0.68
Peripheral vascular disease	7 (2.3%)	9 (4.5%)	0.17
Atrial fibrillation	30 (10%)	19 (10%)	0.87
Smoking	57 (24%)	67 (33%)	0.0001
ECG changes			
ST elevation	17 (5.9%)	74 (36%)	0.001
ST depression	24 (4.9%)	39 (19%)	0.001
Admission medications			
Aspirin	204(68%)	151 (76%)	0.035
B-blocker	134 (45%)	94 (47%)	0.19
ARB	44 (15%)	47 (24%)	0.009
ACE inhibitor	99 (33%)	59 (30%)	0.16
Statin	198 (67%)	135 (68%)	0.20
Discharge medications			
Aspirin	87 (40%)	175 (88%)	0.001
B-blocker	61 (28%)	133 (65%)	0.001
ARB	58 (27%)	28 (14%)	0.002
ACE inhibitor	47 (22%)	91 (45%)	0.001
Statin	108 (50%)	180 (89%)	0.001
Blood results			
Creatinine	76±64	97±64	0.003
LDL cholesterol	2.2±1.1	3±1.1	0.0001
Hemoglobin	125±26	138±18	0.0001
Platelets (mean±SD)	246±92	228±65	0.99
White cell count	8.5±3.4	10±4.3	0.0001
Outcomes			
Heart failure	7 (2.4%)	7 (3.4%)	0.48
Death	1 (0.3%)	3 (1.5%)	0.26
Stroke	0 (0%)	6 (3%)	0.009
Major bleeding	3 (1%)	8 (4.2%)	0.046

ARB, Angiotensin II receptor blocker, COPD, Chronic obstructive pulmonary disease; LDL, low-density lipoprotein; MINOCA, myocardial infarction with nonobstructive coronary arteries; NACE, net adverse cardiovascular events; OACD-MI, obstructive atherosclerotic coronary artery disease myocardial infarction.

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However, death and heart failure incidence were not different among women diagnosed with MINOCA and OACD-MI (Table 2).

On multivariate analysis, presence of female sex, absence of diabetes, previous history of MI, current smoking, and absence of significant ST elevation or ST depression on ECG were the clinical factors associated with diagnosis of MINOCA in the whole cohort of patients presenting with a diagnosis of MI (Table 3).

Sex differences

Out of 645 patients diagnosed with MINOCA, there were 299 women (46%). Compared to men (60 ± 12 years), women diagnosed with MINOCA were older (64 ± 12 years, $P<0.0001$) (Table 4). There were significant differences in the ST-segment patterns of the ECG. Women with MINOCA had less ST elevation on ECG (13 vs. 5.9%, $P=0.002$) and ST depression (8 vs. 4.9%, $P=0.08$) on ECG. There was no difference in cardiac risk factors, prevalence of atrial fibrillation, cancer, and noncardiac illness. Women had lower creatinine levels, higher platelet count and LDL levels. There was no difference in NACE (2.9 vs. 2.9%, $P=0.82$), death (0.3 vs. 0.3%, $P=0.91$), stroke (0.3 vs. 0%, $P=0.35$), or heart failure (2.9 vs. 2.4%, :$P=0.67$). Women had higher access site bleeding ($P=0.03$).

Discussion

In a combined analysis of the two registries, we found MINOCA is found in 9.5% of patients undergoing coronary angiography for a diagnosis of MI. Compared to OACD-MI, patients with MINOCA are more likely to be women, less likely to have traditional reversible cardiac risk factors, less likely to have prior history of cardiovascular disease and tend to have fewer ST changes on ECG. MINOCA patients had a relatively favorable but not a completely benign in-hospital stay with a lower event of NACE. In the MINOCA cohort, women were older than men, had fewer ST changes on the ECG and had higher in-hospital major bleeding events.

The incidence of MINOCA varies significantly among studies because of variability in the definition and differences in the populations studied [7]. Our findings are similar to the data from a large subset of CRUSADE quality improvement initiative which demonstrated the incidence of MINOCA to be around 9% [10]. In the HORIZONS AMI study, the incidence of MINOCA was only 3.5% because only ST-elevation MI patients were analyzed [11]. The incidence of MINOCA has been found to be as high as 15% from registry studies analyzing coronary angiography reports [12]. As shown in Fig. 1, we used a very stringent definition of MINOCA and re-evaluated all angiograms and clinical charts of included patients, so that there were no other clinical findings to suggest alternative causes for elevated cardiac biomarkers. However,

Table 4 Sex differences among patients diagnosed with myocardial infarction with nonobstructive coronary arteries

	Men ($n=346$)	Women ($n=299$)	*P* value
Age (years) (mean ± SD)	60.1 ± 12	64.4 ± 12	<0.0001
History			
Hypertension	195 (56%)	166 (55%)	0.79
Diabetes mellitus	68 (20%)	51 (17%)	0.39
Congestive heart failure	6 (1.8%)	8 (2.7%)	0.42
Myocardial infarction	19 (13%)	7 (5%)	0.01
Stroke	23 (6.7%)	17 (5.7%)	0.61
Cancer	7 (2%)	11 (3.7%)	0.21
Dementia	2 (0.6%)	1 (0.3%)	0.64
COPD	18 (5.2%)	27 (9%)	0.06
Peripheral vascular disease	4 (1.2%)	7 (2.3%)	0.25
Atrial fibrillation	33 (10%)	30 (10%)	0.84
Smoking	82 (19%)	57 (24%)	0.15
Admission ECG			
ST elevation on ECG	43 (13%)	17 (5.9%)	0.002
ST depression on ECG	16 (8%)	24 (4.9%)	0.08
Admission medications			
Aspirin	235 (68%)	204 (69%)	0.92
Warfarin	15 (4.4%)	19 (6.4%)	0.26
B-blocker	160 (47%)	134 (45%)	0.69
ARB	54 (16%)	44 (15%)	0.73
ACE inhibitor	123 (36%)	99 (33%)	0.49
Statin	236 (68%)	198 (67%)	0.64
Discharge medications			
Aspirin	122 (45%)	87 (40%)	0.29
B-blocker	98 (36%)	61 (28%)	0.07
ARB	71 (26%)	58 (27%)	0.83
ACE inhibitor	72 (26%)	47 (22%)	0.24
Statin	156 (57%)	108 (50%)	0.11
Blood results			
Creatinine (mean ± SD), μmol/L	96 ± 83	76 ± 64	0.0001
LDL cholesterol (mean ± SD), mmol/L	1.92 ± 1.2	2.2 ± 1.1	0.02
Hemoglobin (mean ± SD), g/dL	137 ± 28	125 ± 26	0.0001
Platelets (mean ± SD), $\times 10^9$ per L	220 ± 72	246 ± 92	0.0002
White cell count (mean ± SD), $\times 10^9$ per L	8.7 ± 3.6	8.5 ± 3.4	0.7
Outcomes			
NACE	10 (2.9%)	9 (2.7%)	0.82
Heart failure	10 (2.9%)	7 (2.4%)	0.67
Death	1 (0.3%)	1 (0.3%)	0.91
Stroke	1 (0.3%)	0 (0%)	0.35
Bleeding	0 (0%)	3 (1%)	0.03

LDL, low-density lipoprotein; NACE, net adverse cardiovascular events.

Table 3 Multivariate analysis to find predictors independently associated with a diagnosis of myocardial infarction with nonobstructive coronary arteries

MINOCA	Odds ratio	SEM	*Z*	*P* value	95% Confidence interval
Sex (male)	0.41	0.10	-3.42	0.001	0.25–0.68
Diabetes	0.29	0.10	-3.49	0.0001	0.14–0.58
Past history of myocardial infarction	0.36	0.13	-2.65	0.008	0.17–0.76
Current smoking	0.37	0.10	-3.63	0.0001	0.21–0.63
ST elevation on ECG	0.14	0.05	-5.60	0.0001	0.07–0.28
ST depression on ECG	0.25	0.10	-3.42	0.001	0.11–0.55
LDL cholesterol level	0.52	0.06	-4.87	0.0001	0.40–0.68

LDL, low-density lipoprotein.

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we were unable to rule out coronary causes of plaque erosion and microvascular dysfunction as intracoronary imaging and physiological assessment of microcirculation was not performed in most of the patients.

The clinical profile of patients with MINOCA matched that of the general population other than a high incidence of AF which was same as that of OACD-MI [13,14]. Conventional cardiovascular risk factors were significantly lower in the MINOCA group on both univariate and multivariate analysis compared to OACD-MI. The prevalence of atrial fibrillation in MINOCA group was 10% and as high as OACD-MI. The rate of atrial fibrillation in our study is very similar to reported rate in other studies of MINOCA [15]. The rate of atrial fibrillation in MINOCA is found to be nearly 10 times that of the general population [15]. This interesting finding raises two hypothesis generating possibilities, that either the atrial fibrillation induces MI through microembolization or a more likely explanation that atrial fibrillation in MINOCA patients is secondary to underlying endothelial dysfunction or physiological impairment of the microcoronary circulation. There is strong evidence that in the absence of epicardial coronary disease, myocardial blood flow in patients with atrial fibrillation is reduced and coronary flow reserve is impaired [16,17]. These findings have been confirmed both invasively and noninvasively in atrial fibrillation patients without any structural heart abnormality or epicardial coronary obstruction [18,19].

In-hospital cardiovascular events were less likely to happen in MINOCA compared to OACD-MI. In-hospital mortality occurred in only 2.3% of patients. Patients with MINOCA had better outcomes in our study compared to other published data [9]. A likely explanation for low event rates may be due to the use of very stringent definition of MINOCA while previously published studies have included other disorders such as spontaneous coronary artery dissection which have high mortality and morbidity [20]. Furthermore, it remains to be investigated if the increased mortality in the MINOCA group is related to noncardiac illnesses similar to reported mortality in patients with secondary Takotsubo syndrome [21].

Although the European Society of Cardiology statement on MINOCA suggests that these patients should be treated with medications commonly used for MI, we observed that patients with MINOCA were less frequently discharged on dual antiplatelet therapy, statins or beta-blocker therapy. Interestingly, this group of patients was also less likely to be started on MI guideline-based medications on admission. It could be simply because MINOCA patients have a lower incidence of ST-elevation and hence delayed administration of guideline-based therapy. It is also possible that there are differences in the symptomatic presentation.

There are some limitations to the current study. Although our study is a retrospective analysis of prospectively collected data, there was less use of intracoronary imaging, intracoronary physiology and underutilization of cardiac MRI to elucidate the specific cause of MINOCA. We believe that routine use of intracoronary imaging would have allowed the diagnosis of plaque erosion or calcium nodule that is not apparent on standard coronary angiography. Although all of the included patients met the universal definition MI, routine use of cardiac MRI as recommended by the American Heart Association and European Society of Cardiology would have confirmed and provided an extent of the MI while permitting the diagnosis of myocarditis [5,7]. However, the expense and limited availability of cardiac MRI are limiting factors. Second, although we have demonstrated differences in outcome in MINOCA vs. OACD-MI, we lack the sample size in order to elucidate differences in outcomes in men or women diagnosed with MINOCA. Finally, our study only reports acute outcomes. We did not have a long-term follow of all the patients to report chronic disease course of the patients with MINOCA. The long-term outcome data would be important to understand the natural history of the disease to develop treatment interventions.

In conclusion, more women are diagnosed with MINOCA than OACD-MI. Female sex, absence of traditional cardiac risk factors and an absence of ST changes on the ECG are associated with MINOCA following angiography. Patients diagnosed with MINOCA had better in-hospital outcomes compared to OACD-MI. There were no significant differences among patients diagnosed with MINOCA when stratified by sex. The association of a high prevalence of atrial fibrillation in patients with MINOCA merits further studies. Lastly, there is underutilization of guideline-based therapy in MINOCA patients and further trials are needed to find the evidence of recommended treatments in this group of patients.

Acknowledgements

R.G.J. was funded by the Vanier CIHR Canada Graduate Scholarship for his graduate studies. The authors would like to thank the CAPITAL Research Group for all their assistance in the completion of the project.

Conflicts of interest

There are no conflicts of interest.

References

1 Bonaca MP, Wiviott SD, Braunwald E, Murphy SA, Ruff CT, Antman EM, Morrow DA. American College of Cardiology/American Heart Association/European Society of Cardiology/World Heart Federation universal definition of myocardial infarction classification system and the risk of cardiovascular death: observations from the TRITON-TIMI 38 trial (Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition With Prasugrel-Thrombolysis in Myocardial Infarction 38). *Circulation* 2012; 125:577–583.

2 Higuma T, Soeda T, Abe N, Yamada M, Yokoyama H, Shibutani S, *et al.* A combined optical coherence tomography and intravascular ultrasound study on plaque rupture, plaque erosion, and calcified nodule in patients

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with ST-segment elevation myocardial infarction: incidence, morphologic characteristics, and outcomes after percutaneous coronary intervention. *JACC Cardiovasc Interv* 2015; **8**:1166–1176.
3 DeWood MA, Stifter WF, Simpson CS, Spores J, Eugster GS, Judge TP, Hinnen ML. Coronary arteriographic findings soon after non-Q-wave myocardial infarction. *N Engl J Med* 1986; **315**:417–423.
4 Thygesen K, Alpert JS, Jaffe AS, Simoons ML, Chaitman BR, White HD, *et al.*; Joint ESC/ACCF/AHA/WHF Task Force for Universal Definition of Myocardial Infarction; Authors/Task Force Members Chairpersons; Biomarker Subcommittee; ECG Subcommittee; Imaging Subcommittee; Classification Subcommittee; Intervention Subcommittee; Trials & Registries Subcommittee; Trials & Registries Subcommittee; Trials & Registries Subcommittee; Trials & Registries Subcommittee; ESC Committee for Practice Guidelines (CPG); Document Reviewers. Third universal definition of myocardial infarction. *J Am Coll Cardiol* 2012; **60**:1581–1598.
5 Agewall S, Beltrame JF, Reynolds HR, Niessner A, Rosano G, Caforio AL, *et al.*; WG on Cardiovascular Pharmacotherapy. ESC working group position paper on myocardial infarction with non-obstructive coronary arteries. *Eur Heart J* 2017; **38**:143–153.
6 Le May MR, So DY, Dionne R, Glover CA, Froeschl MP, Wells GA, *et al.* A citywide protocol for primary PCI in ST-segment elevation myocardial infarction. *N Engl J Med* 2008; **358**:231–240.
7 Tamis-Holland JE, Jneid H, Reynolds HR, Agewall S, Brilakis ES, Brown TM, *et al.* Contemporary diagnosis and management of patients with myocardial infarction in the absence of obstructive coronary artery disease: a scientific statement from the American Heart Association. *Circulation* 2019; **139**:e891–e908.
8 Ezekowitz JA, O'Meara E, McDonald MA, Abrams H, Chan M, Ducharme A, *et al.* 2017 comprehensive update of the Canadian cardiovascular society guidelines for the management of heart failure. *Can J Cardiol* 2017; **33**:1342–1433.
9 Pasupathy S, Air T, Dreyer RP, Tavella R, Beltrame JF. Systematic review of patients presenting with suspected myocardial infarction and nonobstructive coronary arteries. *Circulation* 2015; **131**:861–870.
10 Patel MR, Chen AY, Peterson ED, Newby LK, Pollack CV Jr, Brindis RG, *et al.* Prevalence, predictors, and outcomes of patients with non-ST-segment elevation myocardial infarction and insignificant coronary artery disease: results from the can rapid risk stratification of unstable angina patients Suppress ADverse outcomes with Early implementation of the ACC/AHA Guidelines (CRUSADE) initiative. *Am Heart J* 2006; **152**:641–647.
11 Larsen AI, Nilsen DW, Yu J, Mehran R, Nikolsky E, Lansky AJ, *et al.* Long-term prognosis of patients presenting with ST-segment elevation myocardial infarction with no significant coronary artery disease (from the HORIZONS-AMI trial). *Am J Cardiol* 2013; 111:643–648.
12 Barr PR, Harrison W, Smyth D, Flynn C, Lee M, Kerr AJ. Myocardial infarction without obstructive coronary artery disease is not a benign condition (ANZACS-QI 10). *Heart Lung Circ* 2018; **27**:165–174.
13 Lacruz ME, Kluttig A, Hartwig S, Löer M, Tiller D, Greiser KH, *et al.* Prevalence and incidence of hypertension in the general adult population: results of the CARLA-cohort study. *Medicine (Baltimore)* 2015; **94**:e952.
14 Catalán-Ramos A, Verdú JM, Grau M, Iglesias-Rodal M, del Val García JL, Consola A, Comin E; @GPC-ICS Group. Population prevalence and control of cardiovascular risk factors: what electronic medical records tell us. *Aten Primaria* 2014; **46**:15–24.
15 Eggers KM, Hjort M, Baron T, Jernberg T, Nordenskjöld AM, Tornvall P, Lindahl B. Morbidity and cause-specific mortality in first-time myocardial infarction with nonobstructive coronary arteries. *J Intern Med* 2019; **285**:419–428.
16 Wijesurendra RS, Casadei B. Atrial fibrillation: effects beyond the atrium? *Cardiovasc Res* 2015; **105**:238–247.
17 Wijesurendra RS, Liu A, Notaristefano F, Ntusi NAB, Karamitsos TD, Bashir Y, *et al.* Myocardial perfusion is impaired and relates to cardiac dysfunction in patients with atrial fibrillation both before and after successful catheter ablation. *J Am Heart Assoc* 2018; **7**:e009218.
18 Kochiadakis GE, Skalidis EI, Kalebubas MD, Igoumenidis NE, Chrysostomakis SI, Kanoupakis EM, *et al.* Effect of acute atrial fibrillation on phasic coronary blood flow pattern and flow reserve in humans. *Eur Heart J* 2002; **23**:734–741.
19 Range FT, Schäfers M, Acil T, Schäfers KP, Kies P, Paul M, *et al.* Impaired myocardial perfusion and perfusion reserve associated with increased coronary resistance in persistent idiopathic atrial fibrillation. *Eur Heart J* 2007; **28**:2223–2230.
20 Safdar B, Spatz ES, Dreyer RP, Beltrame JF, Lichtman JH, Spertus JA, *et al.* Presentation, clinical profile, and prognosis of young patients with myocardial infarction with nonobstructive coronary arteries (MINOCA): results from the VIRGO study. *J Am Heart Assoc.* 2018; **7**:e009174.
21 Singh K, Carson K, Shah R, Sawhney G, Singh B, Parsaik A, *et al.* Meta-analysis of clinical correlates of acute mortality in Takotsubo cardiomyopathy. *Am J Cardiol* 2014; **113**:1420–1428.

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Appendix K

Evaluation of plasma adenosine as a marker of cardiovascular risk: analytical and biological considerations

Citation information:

Jung RG*, Simard T*, Labinaz A, Faraz MA, Ramirez FD, Di Santo P, Perry-Nguyen D, Pitcher I, Motazedian P, Gaudet C, Rochman R, Marbach J, Boland P, Sarathy K, Alghofaili S, Russo JJ, Couture E, Promislow S, Beanlands RS, Hibbert B. Circulating adenosine as a biomarker of cardiovascular outcomes: technical and biological considerations. **(2019).** *Journal of American Heart Association.*

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ORIGINAL RESEARCH

Evaluation of Plasma Adenosine as a Marker of Cardiovascular Risk: Analytical and Biological Considerations

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Background—Adenosine is a ubiquitous regulatory molecule known to modulate signaling in many cells and processes vital to vascular homeostasis. While studies of adenosine receptors have dominated research in the field, quantification of adenosine systemically and locally remains limited owing largely to technical restrictions. Given the potential clinical implications of adenosine biology, there is a need for adequately powered studies examining the role of plasma adenosine in vascular health. We sought to describe the analytical and biological factors that affect quantification of adenosine in humans in a large, real-world cohort of patients undergoing evaluation for coronary artery disease.

Methods and Results—Between November 2016 and April 2018, we assessed 1141 patients undergoing angiography for evaluation of coronary artery disease. High-performance liquid chromatography was used for quantification of plasma adenosine concentration, yielding an analytical coefficient of variance (CV_a) of 3.2%, intra-subject variance (CV_i) 35.8% and inter-subject variance (CV_g) 56.7%. Traditional cardiovascular risk factors, medications, and clinical presentation had no significant impact on adenosine levels. Conversely, increasing age (P=0.027) and the presence of obstructive coronary artery disease (P=0.026) were associated with lower adenosine levels. Adjusted multivariable analysis supported only age being inversely associated with adenosine levels (P=0.039).

Conclusions—Plasma adenosine is not significantly impacted by traditional cardiovascular risk factors; however, advancing age and presence of obstructive coronary artery disease may be associated with lower adenosine levels. The degree of intra- and inter-subject variance of adenosine has important implications for biomarker use as a prognosticator of cardiovascular outcomes and as an end point in clinical studies. **(*J Am Heart Assoc.* 2019;8:e012228. DOI: 10.1161/JAHA.119.012228.)**

Key Words: adenosine • biomarker • coronary artery disease • high-performance liquid chromatography • plasma

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Adenosine is a purine nucleoside that serves as a crucial intracellular and extracellular regulatory molecule regulating numerous blood and vascular cell types.[1,2] The metabolism of adenosine is regulated by a close balance of production, transport (primarily via equilibrative nucleoside transporters—ENTs) and degradation (primarily via adenosine deaminase—ADA).[3–5] Adenosine circulating in the extracellular space signals mainly via P1 purinergic receptors, G-protein-coupled receptors with differential responses to adenosine depending on which of the 4-subtypes of adenosine receptors (ADOR) are stimulated—ADORA1, ADORA2A, ADORA2B, and ADORA3.[6] Numerous preclinical studies have suggested adenosine regulates vascular homeostasis, with regulatory implications for inflammatory cells, smooth muscle cells, endothelial cells and platelets.[7–12] However, in humans little is known about variance of plasma adenosine concentration (PAC) or factors which influence PAC owing to technical challenges in quantifying levels in large cohorts of patients.

Therapeutically, adenosine's clinical applications have been relatively focused. Intravenous adenosine boluses are predominantly used as a diagnostic and therapeutic agent in

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Accompanying Tables S1 through S5 and Figures S1, S2 are available at https://www.ahajournals.org/doi/suppl/10.1161/JAHA.119.012228

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Received January 30, 2019; accepted May 24, 2019.

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Clinical Perspective

What Is New?

- Our robust assessment in a large, real-world cohort of patients undergoing evaluation for coronary artery disease demonstrated considerable biologic variability in circulating adenosine levels.
- Advancing age may be associated with reduced circulating adenosine levels, while cardiovascular risk factors and medications did not significantly impact levels.

What Are the Clinical Implications?

- The biologic variability and clinical factors influencing circulating adenosine levels should be considered when using adenosine as an end point in clinical studies or as a predictor of cardiovascular outcomes.

the management of tachyarrhythmias. Secondarily, adenosine and agents that augment PAC have been used for induction of coronary hyperemia for flow-related non-invasive and invasive assessment of myocardial perfusion.[13] Dipyridamole, acting primarily via ENT inhibition to augment adenosine levels, is more broadly employed for its flow-mediated effects and less commonly as an anti-platelet agent.[14] To minimize off target effects, small molecule agents have been developed to target specific adenosine receptors, such as regadenoson (an ADORA2A specific agonist) for maximizing coronary vasodilation.[15] Nonetheless, despite promising preclinical studies, a translational gap exists whereby the therapeutic application of adenosine modulation has been hampered by complex receptor biology and a limited understanding of adenosine levels in human disease pathogenesis.

Clinically, the measurement of circulating adenosine has seen limited use—though some studies have either reported prognostic significance in small cohorts or used PAC as an end point in clinical trials.[16,17] Quantification of PAC by high-performance liquid chromatography (HPLC) is an established methodology with reported analytical variability (CV_a) ranging from 6% to 7%[18] and up to 10%[19] previously, with more contemporary assays yielding CVs of 1% to 3%, in keeping with clinical assay standards.[20] With these protocols, some small studies (n=10) have demonstrated reduced local circulating adenosine levels via coronary sampling immediately following balloon angioplasty for coronary artery disease (CAD).[21] Others report elevated PAC in patients (n=71) with chronic congestive heart failure (CHF), proposing it provides protective effects from rising norepinephrine levels.[22] Interestingly, genetic studies in patients with adenosine monophosphate deaminase locus 1 (AMPD1) mutations (putatively augmenting adenosine levels) demonstrate improved survival in CHF patients.[23,24] AMPD1 carriers have also demonstrated improved cardiovascular survival in those with angiographically documented CAD,[25] though this did not hold true for patients post-revascularization.[26] While all of these studies invoke an adenosine-mediated mechanism, definitive links between atherosclerotic risk factors, disease burden and adenosine levels have yet to be established.[2]

Given the prognostic and therapeutic implications of adenosine levels and the lack of robust human data, we set out to systematically evaluate the analytic characteristics of PAC quantification and determine if traditional cardiac risk factors, cardiac therapies, and/or disease burden are associated with PAC.

Methods

Adenosine Sample Collection and Processing

Blood samples were collected at the time of angiography via a 6-French plastic arterial access sheath (Terumo Medical, Somerset, NJ) placed in the radial artery. Rarely, if this was not possible, then venous samples were collected via peripheral venipuncture. Blood samples (6 mL) were collected in Greiner BioOne Vacuette tubes pre-injected with 2 mL of ice-cold stop solution. Stop solution was composed of 100 μmol/L dipyridamole, 2.5 μmol/L erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), 1 U/mL heparin in 0.9% saline. Tubes were inverted and connected to the access sheath to ensure rapid and direct mixing of blood with stop solution and were maintained on ice before and following draws until processing. Samples were centrifuged at 4°C, 1200*g* for 10 minutes without brakes to limit platelet activation and the supernatant was collected and stored at −80°C until processing. Hemolyzed samples (which result in markedly elevated PAC levels) were excluded on a biological basis in keeping with prior studies.[18] The data that support the findings of this study are available from the corresponding author upon reasonable request.

Aliquots were then thawed at room temperature and centrifuged at 1000*g* for 3 minutes and 500 μL of sample was diluted in 500 μL of 4% phosphoric acid. This 1 mL combined solution was then loaded onto a Waters Oasis MCX (Mixed-mode, strong Cation-eXchange) 1 mL cartridge and the sample was eluted with vacuum assistance through the column as per protocol. The sample was then washed with 1 mL of 2% formic acid followed by 500 μL of 100% methanol with vacuum assistance between each wash. The final sample was then eluted using 2 sequential 125 μL elutions with MCX eluting solution (5% NH_4OH in 60/40 acetonitrile/methanol) followed by vacuum assistance to ensure all samples were collected from the vial. Samples were then transferred to vials to undergo HPLC analysis. Adenosine standards were

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prepared using pharmaceutical grade adenosine (Sigma PHR11380-1G) diluted in MCX eluting solution.

Samples were analyzed using HPLC on a Waters Alliance E2695 separating module system with sample quantification by Waters 2489 UV/visible detector at 260 nm. The mobile phase was composed of a mixture of Mobile Phase A (10 μmol/L ammonium formate pH3 in 50% acetonitrile:50% water, ranging 1–50%) and Mobile Phase B (10 μmol/L Ammonium Formate pH3 in 95% Acetonitrile:5% Water, ranging 50–99%) with a sample temperature of 4°C and column temperature of 24°C. Samples were then processed through a Waters Xbridge BEH amide SP Vanguard Cartridge pre-column and subsequent Waters Xbridge BEH Amide 2.5 μm, 4.6×150 mm column XP. Data processing was completed using Waters Empower 3 Software.

Assay Validation for Plasma Adenosine Quantification

Quantification of variance is achieved via relative standard deviation (RSD) and the coefficient of variation (CV). Both RSD and CV are percentages representing the standard deviation (SD) divided by the mean value to standardize the variability for a given result. RSD is an absolute value, while CV is not. We report CV in keeping with prior studies.[27] The CV is assessed at multiple stages of our assay and defined accordingly (1) CV_a, CV analytical, the variation of the HPLC assay itself including the processing and analysis of samples (generated via multiple aliquots obtained from a single tube drawn from a single patient), (2) CV_i, CV individual, the intra-subject variation over time generated from serial samples from the same patient collected on different days via $CV_i = (CV_t^2 - CV_a^2)^{1/2}$ and (3) CV_g, CV group, the inter-subject variation within the population of subjects studied (generated from different samples collected from different patients at different times).[28,29] The reference change value (RCV) was calculated via $2.77\ (CV_a^2 + CV_i^2)^{1/2}$, while the index of individuality (II) was calculated by $(CV_a^2 + CV_i^2)^{1/2}/CV_g$, in keeping with prior reports.[28–31] The validation of our HPLC methodology followed good practice guidelines as published previously.[32] Specificity of the assay was maximized by adjusting the gradients and temperatures until adequate separation of the adenosine peak of interest was achieved from the surrounding peaks. The specific identity of the adenosine peak was confirmed by focused degradation of adenosine by ADA followed by quantification to demonstrate loss of the adenosine peak (Figure—Panel A). Repeatability was assessed in both the standards and samples to determine the intra-day assay precision using the same conditions. Standards in eluting solution were injected 10 sequential times from the same vial, while samples prepared with the MCX system were injected 6 sequential times from the same vial. Both the retention times and peak areas were recorded for each run and a mean, SD, and CV reported (Table S1). Linearity and range were assessed by creating 3 individual sample preparations of standards ranging in concentration from 100 to 15 000 nmol/L representing 10% to 1500% of the target concentration of adenosine (1000 nmol/L) (Figure—Panel B, Table S2). These individual samples were run on the same machine on the same day to generate the appropriate curves from which the retention time and peak areas were recorded across each individual preparation with means, standard deviation (SD) and CV then calculated (Table S2). The adenosine standard curve (Figure—Panel B) was then assessed for linearity over a range from 100 to 15 000 nmol/L adenosine concentrations in both elution buffer and matrix (adenosine-depleted plasma generated via ADA degradation of endogenous adenosine).

Ongoing data validation during the sample collection phase was ensured by repetition of a standardized protocol including blank injection before, in the middle of, and following sample injections. Similarly, a blank phosphate-buffered saline sample is processed through the MCX column and quantified. Standards are run with each grouping of samples and the curves are monitored for stability including slope, intercept and R^2. The stability of samples during the HPLC analysis period is ensured by performing 3 injections of a given sample at the start, middle and end of each run to ensure consistent results. We also use 1 sample with which we perform (1) ADA degradation, (2) ADA degradation followed by adenosine spiking post MCX column, and (3) adenosine spiking pre- and post-MCX columns. This process is then repeated in phosphate-buffered saline with both an adenosine spike and an adenosine spike followed by ADA degradation. In this way, constant monitoring of the quality and reliability of results generated is ensured over time.[32]

Biological Sample and Clinical Data Collection

The University of Ottawa Heart Institute is a high volume, tertiary care center providing coronary revascularization services to >1.2 million people.[33] From November 2016 to April 2018, 7252 patients were prospectively enrolled in the CAPITAL (Cardiovascular And Percutaneous Clinical Trials) revascularization registry which indexes clinical data points on patients undergoing coronary angiography and revascularization. In the CAPITAL revascularization registry, coronary artery disease (CAD) was defined as obstructive stenosis ≥50% at the time of angiography in keeping with current clinical standards.[34] Acute coronary syndrome (ACS) was composed of troponin-positive presentations including both non–ST-segment–elevation myocardial infarction and

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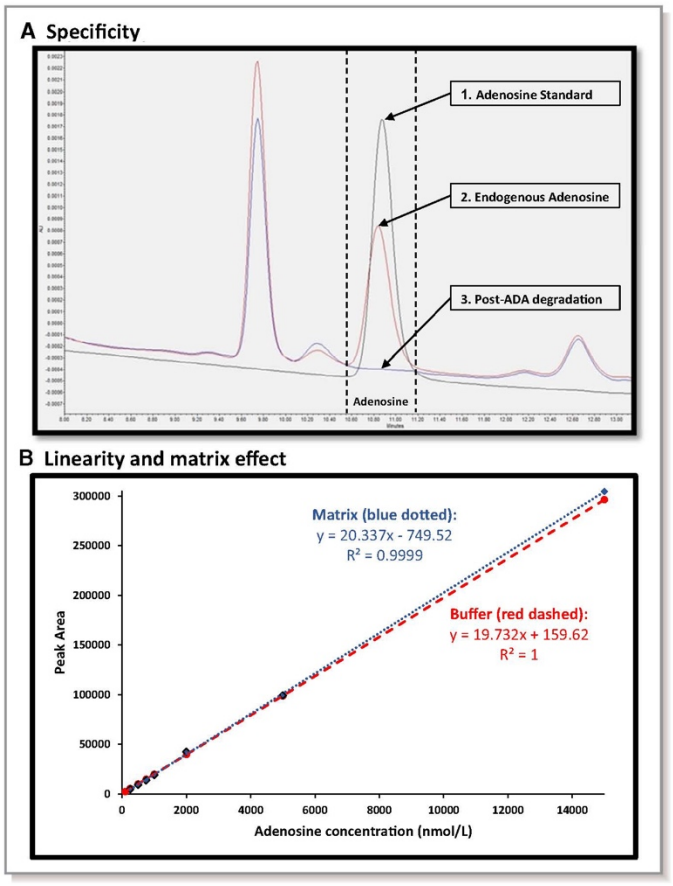


Figure. Validation of adenosine high-performance liquid chromatography methodology. **A**, Superimposed chromatograms of 3 separate samples demonstrating a distinct adenosine peak free of interfering peaks. Multiple superimposed peaks including (1) adenosine standard in elution buffer, (2) endogenous adenosine in plasma (matrix) sample, and (3) endogenous plasma sample following degradation of adenosine with adenosine deaminase resulting in no detectable adenosine peak confirming peak specificity. **B**, Curve generated by plotting peak areas by adenosine concentration for both standards in buffer (dashed red line) and standards in plasma sample matrix (dotted blue line) demonstrating excellent linearity and minimal matrix effect. ADA indicates adenosine deaminase.

ST-segment–elevation myocardial infarction cases. Diabetes mellitus (DM) was based on either a hemoglobin A1c (HbA1c) ≥6.5% on presentation or a prior DM diagnosis or presence of medical therapy for DM. Tobacco use was dichotomized into smokers (active smoking at the time of sample collection) or non-smokers (not smoking at the time of sample collection). Positive family history was defined as CAD in a first-degree relative aged <55 years for men and <65 years for women. Dyslipidemia and hypertension were defined as either a prior diagnosis of either condition or the presence of the appropriate medical therapy for either diagnosis on presentation. This study received approval from the University of Ottawa Heart Institute ethics review board (Protocols #20180562-01H, #20160516-01H and #20170126-01H) and informed consent was completed. Of this cohort of patients undergoing evaluation for coronary artery disease, 1174 patients had blood samples collected for analysis.

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Statistical Methods

Data are reported as mean±SD, median±interquartile range or number and percentage (%) where appropriate. Statistical testing was completed using GraphPad Prism 7.04, SigmaStat and SAS v9.4. Biological data were assessed for normality using D'Agostino and Pearson or Shapiro-Wilk normality tests. Following log transformation of the data set, no statistical outliers were identified. Comparisons of 2 groups of non-parametric data were performed using Mann–Whitney test. All analyses defined significance as a 2-tailed $P<0.05$, unless otherwise specified. Log-transformation of all data was completed before regression analysis. Linear regression was performed for age with demonstrated 95% confidence intervals. Univariable linear regression was similarly performed for all factors with a predetermined $P<0.2$ used to identify factors for inclusion in subsequent multivariable linear regression analysis with significance defined as $P<0.05$ in keeping with prior studies.[35]

Results

HPLC methodology validation and analytical variability

Robust assay specificity was demonstrated generating a discernible adenosine peak free of interference from surrounding peaks. Focused degradation of adenosine by ADA demonstrated complete abrogation of the adenosine peak (Figure—Panel A). Repeatability of the HPLC assay itself was assessed by sequential injections from the same preparation of (1) standard (10 sequential injections on the same day of 1000 nmol/L adenosine, representing 100% target concentration) and (2) sample (6 sequential injections on the same day of serum from a single subject). This approach demonstrated a CV for retention time, peak area, and peak height of 0.12%, 2.19%, and 0.65% for standards and 0.16%, 1.52%, and 1.96% for samples, respectively (Table S1). Our assessment of linearity and range included reporting the retention time and peak area for all adenosine values ranging from 100 to 15 000 nmol/L (10–1500% of target)—producing CV ranging from 0.02% to 0.22% for retention time, while the CV for peak areas ranged from 0.78% to 7.03% at 250 nmol/L (Table S2). The lowest quantified value, 100 nmol/L of adenosine, demonstrated a variance of 12.87% identifying a lower limit for reliable quantification by this assay. Plotting the peak areas generated as a function of the adenosine concentrations generates a line of best fit with equation $y=19.732x+159.62$, $R^2=1$ (Figure—Panel B, dashed line). The impact of matrix (plasma) was assessed with the same range of standard concentrations and compared with the curve generated with standards in elution buffer on the same plot (Figure—Panel B, dotted line), resulting in a trendline of $y=20.337x-749.52$, $R^2=0.9999$.

Intra and inter-subject variability

The variability of our assay and methodology was assessed at each level of quantification in an unselected cohort of patients undergoing assessment for coronary artery disease (Table 1). First, intra-tube variability was assessed using multiple aliquots from the same blood tube drawn from 1 subject demonstrating a CV of 3.2%—establishing the analytical CV (CV_a) for our assay. Next, intra-subject variability was assessed, first for inter-tube variability using separate draws at the same time point yielding a CV of 23.0%. Intra-subject variation was then assessed within the same day and on separate days. Adenosine levels drawn throughout a single day incrementally increased the CV to 30.1%, while serial collections on the same patient across multiple days (mean 35.8±33.1 days) further increased the CV to 35.8%—establishing the CV_i for our assay. Lastly, inter-subject variation (CV_g) was assessed using all adenosine levels in the entire cohort, noting a CV_g of 56.7% (Table S3). Overall, this resulted in a RCV of 98.9% and an II of 0.63.

Patient and procedural characteristics

From an initially recruited 1174 patients, we excluded duplicate samples of the same patients (33 in total) leaving 1141 patients included in the final analysis. The cohort's baseline demographics are summarized in Table 2. The

Table 1. Intra- and Inter-Subject Variation

	No. of Subjects	No. of Samples	Adenosine (nmol/L)	SD (nmol/L)	CV (%)
Intra-subject					
Intra-tube, same time (CV_a)	18	92	1042.0	33.5	3.2
Inter-tube, same time	29	95	1240.0	256.1	23.0
Different time, same day	17	39	1090.8	349.3	30.1
Different time, different day (CV_i)	31	64	1216.6	457.3	35.8
Inter-subject					
Different time, different day (CV_g)	1141	…	1067.2	605.3	56.7

CV indicates coefficient of variation; CV_a, analytical CV; CV_g, inter-subject CV; CV_i, intra-subject CV; SD, standard deviation.

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average age was 66.3±11.8 years (Figure S1A) with 70.8% being male. The cohort underwent angiography for indications that included ACS (39.5%) and stable CAD (39.9%). Risk factors included 30.4% with diabetes mellitus, 18.5% active smokers, 61.3% with dyslipidemia, 16.5% with positive family history, and 64.7% with hypertension. Coronary artery disease was known before angiography in 38.1% patients, with 24.7% reporting a prior myocardial infarction and 34.6% having had prior revascularization with either percutaneous coronary intervention or coronary artery bypass grafting. Medical therapy in the cohort included 53.5% on angiotensin-converting enzyme inhibitors/ARB (Angiotensin II receptor blocker), 59.4% on beta blockers, 79.7% on statins, 88.4% on aspirin and 87.7% on P2Y12 inhibitors. A total of 25 recruited patients did not undergo angiography, but had samples collected via venous access, leaving 1116 patients who underwent angiography for which procedural details were indexed (Table 3). After excluding those with prior revascularization, CAD, or myocardial infarction, 633 cases remained that underwent angiography, of which 431 cases remained that had de novo obstructive CAD at the time of sample collection—ranging from 1 vessel (40.1%), 2 vessel (28.5%) to 3 vessel (31.3%) disease. Of the entire cohort, 23.5% underwent percutaneous coronary intervention with placement of 1 stent (46.2%), 2 stents (32.4%) or ≥3 stents (21.4%).

Impact of Cardiovascular Risk Factors on PAC

Established cardiovascular risk factors were assessed for impact on circulating adenosine levels (Table 4). Smokers did not show a statistical difference in adenosine levels compared with non-smokers (917 [607–1325] nmol/L versus 932 [635–1357] nmol/L, P=0.858). As well, there was no statistical difference in adenosine levels between those that did and did not have a history of dyslipidemia (909 [626–1350] versus 953 [645–1390] nmol/L, P=0.292), hypertension (936 [634–1363] versus 917 [621–1353] nmol/L, P=0.701), or family history of CAD (953 [609–1376] versus 926 [635–1350] nmol/L, P=0.896). Sex did not impact adenosine levels with males (925 [630–1345] nmol/L) (demonstrating similar levels to females (949 [645–1398] nmol/L, P=0.293). Diabetes mellitus as a dichotomized variable did not significantly impact adenosine levels with diabetics and non-diabetics (974 [604–1438] versus 913 [639–1313] nmol/L, P=0.238). Moreover, in the 294 diabetic patients with HbA1c values available, there was no significant relationship between HbA1c and adenosine levels (r=0.03, R^2=0.001, P=0.59, Figure S2A). The impact of age on adenosine levels was also assessed (Figure S2B) showing a statistically significant inverse association between age and PAC (R^2=0.005, r=−0.07, P=0.02). Next, we performed additional analysis following division of the cohort into those aged ≤65 (n=533)

Table 2. Baseline Demographics

	Number or Mean	Proportion (%) or Standard Deviation
Number of patients	1141	…
Age	66.3	11.8
Male	806	70.8
Indication for angiography		
Acute coronary syndrome	451	39.5
STEMI	24	2.1
NSTEMI	292	25.6
Unstable angina	135	11.8
Stable coronary artery disease	455	39.9
Staged PCI	124	10.9
Shock	2	0.2
Arrhythmia	19	1.7
Heart failure/LV dysfunction	90	7.9
Past medical history		
Diabetes mellitus	347	30.4
Type I	7	2.0
Type II—diet controlled	13	3.7
Type II—non-insulin therapy	233	67.1
Type II—insulin therapy	94	27.1
Smoking	211	18.5
Dyslipidemia	699	61.3
Family history	188	16.5
Hypertension	738	64.7
Prior cerebrovascular accident	80	7.0
Peripheral arterial disease	84	7.4
Atrial fibrillation	119	10.4
Prior coronary artery disease	435	38.1
Prior myocardial infarction	282	24.7
Prior angiogram	462	40.5
Prior PCI	317	27.8
Prior coronary artery bypass grafting	78	6.8
Medications		
ACE inhibitor/ARB	610	53.5
Beta blocker	678	59.4
Calcium channel blocker	158	13.8
Statin	909	79.7
Oral anticoagulation	61	5.3
Intravenous unfractionated heparin	132	11.6
Subcutaneous LMWH	123	10.8
Acetylsalicylic acid	1009	88.4

Continued

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Table 2. Continued

	Number or Mean	Proportion (%) or Standard Deviation
P2Y12	1001	87.7
Clopidogrel	685	68.4
Ticagrelor	315	31.5
Prasugrel	1	0.1

ACE indicates angiotensin-converting enzyme; ARB, angiotensin receptor blocker; LMWH, low-molecular weight heparin; LV, left ventricular; PCI,percutaneous coronary intervention; SD, standard deviation; STEMI, ST-segment–elevation myocardial infarction; NSTEMI, Non–ST-segment–elevation myocardial infarction.

and those aged >65 (n=608) years, demonstrating reduced adenosine in the >65-year cohort (895 [610–1315] nmol/L) than the ≤65-year cohort (971 [649–1397] nmol/L, *P*=0.027).

Impact of Medical Therapy on Plasma Adenosine Levels

Medical therapy for cardiovascular risk reduction was assessed for impact on PAC (Table 4). No difference in adenosine levels was noted comparing patients taking to those not taking angiotensin-converting enzyme inhibitors/ARBs (943 [645–1353] nmol/L versus 909 [620–1363] nmol/L, *P*=0.419) beta blockers (958 [644–1356] nmol/L versus 887 [615–1357] nmol/L, *P*=0.317) calcium channel blockers (974 [618–1398] nmol/L versus 919 [634–1346] nmol/L, *P*=0.433) and statins (919 [618–1341] nmol/L versus 937 [690–1419] nmol/L, *P*=0.141). Similarly, anticoagulants used preceding angiography did not impact adenosine levels with unfractionated heparin (985 [619–1397] versus 925 [634–1350] nmol/L, *P*=0.549) or subcutaneous low-molecular weight heparin (907 [654–1400] versus 932 [630–1350] nmol/L, *P*=0.568). Finally, we evaluated the impact of antiplatelet therapy on PAC as previous data suggesting ticagrelor may impact adenosine metabolism (Table 4).[16,17] No difference in adenosine was seen with acetylsalicylic acid (ASA) (919 [626–1361] versus 958 [683–1285] nmol/L, *P*=0.65). Similarly, in the class of P2Y12 inhibitors, clopidogrel did not affect adenosine levels (953 [637–1400] versus 904 [609–1289] nmol/L, *P*=0.158). Interestingly, ticagrelor therapy was associated with a reduction in adenosine levels compared with those not on ticagrelor (875 [595–1254] versus 955 [650–1408] nmol/L, *P*=0.012).

Impact of Coronary Artery Disease on Plasma Adenosine Levels

Finally, we assessed the impact of CAD presence and burden on plasma adenosine levels (Table 4). In the total cohort, absence of obstructive CAD was associated with higher adenosine levels than patients with obstructive CAD (909 [618–1325] versus 995 [686–1460] nmol/L, *P*=0.026). No differences between patients presenting as ACS (non–ST-segment–elevation myocardial infarction /ST-segment–elevation myocardial infarction) versus non-ACS and were observed (n=311, 932 [637–1346] nmol/L versus n=830, 928 [630–1363] nmol/L, *P*=0.971). Disease burden, as assessed by presence of de novo multivessel (>1 vessel) disease, failed to show any association with PAC levels compared with those with single-vessel disease (902 [616–1306] versus 926 [604–1356] nmol/L, *P*=0.676).

Table 3. Procedural Details

	Number or Mean	Proportion (%) or Standard Deviation
Number of patients undergoing angiography	1116	…
Access		
Radial	1015	90.9
Femoral	97	8.7
Brachial	4	0.4
Access site medications		
Calcium channel blocker	585	52.4
Nitroglycerin	420	37.6
Procedural medications		
Heparin	966	88.1
Mean dose (U)	6678	2378
Bivalirudin	53	4.7
Glycoprotein IIb/IIIa inhibitors	2	0.2
Adenosine	68	6.1
Intravenous	30	2.7
Intracoronary	36	3.2
Nitroglycerin	457	40.9
Number of cases with de novo obstructive (≥50%) CAD	431	38.6
Lesion-burden		
1 lesion	121	28.1
2 lesions	97	22.5
3 lesions	68	15.8
4 lesions	59	13.7
≥5 lesions	88	20.4
Vessel-burden		
1 vessel	173	40.1
2 vessel	123	28.5
3 vessel	135	31.3
Number of cases with a stent deployed	262	23.5
1 stent	121	46.2
2 stents	85	32.4
≥3 stents	56	21.4

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Table 4. Impact of Risk Factors, Medications, and Coronary Artery Disease on Adenosine

	Present		Absent		
	n	Median (IQR) (nmol/L)	n	Median (IQR) (nmol/L)	*P* Value
Cardiovascular risk factors					
Age >65 y	608	895 (610–1315)	533	971 (649–1397)	0.027*
Diabetes mellitus	347	974 (604–1438)	793	913 (639–1313)	0.238
Smoking	211	917 (607–1325)	930	932 (635–1357)	0.858
Dyslipidemia	699	909 (626–1350)	442	953 (645–1390)	0.292
Family history	188	953 (609–1376)	953	926 (635–1350)	0.896
Hypertension	738	936 (634–1363)	403	917 (621–1353	0.701
Male	806	925 (630–1345)	335	949 (645–1398)	0.293
Medications					
Acetylsalicylic acid	1023	919 (626–1361)	117	958 (683–1285)	0.650
Clopidogrel	685	953 (637–1400)	456	904 (609–1289)	0.158
Ticagrelor	315	875 (595–1254)	826	955 (650–1408)	0.012*
ACE inhibitor/ARB	610	943 (645–1353)	531	909 (620–1363)	0.419
Beta blocker	678	958 (644–1356)	463	887 (615–1357)	0.317
Calcium channel blocker	158	974 (618–1398)	983	919 (634–1346)	0.433
Statin	909	919 (618–1341)	232	937 (690–1419)	0.141
Unfractionated heparin	132	985 (619–1397)	1009	925 (634–1350)	0.549
Low-molecular weight heparin	123	907 (654–1400)	1018	932 (630–1350)	0.568
Coronary artery disease					
CAD	941	909 (618–1325)	200	995 (686–1460)	0.026*
De novo CAD burden >1 vessel	548	902 (616–1306)	315	926 (604–1356)	0.676
Acute coronary syndrome	311	932 (637–1346)	830	928 (630–1363)	0.971

ACE indicates angiotensin-converting enzyme; ARB, Angiotensin II receptor blocker; CAD, coronary artery disease; IQR, interquartile range.
*$P<0.05$.

Multivariable Linear Regression Analysis

To assess the association of variables with PAC, we first performed a log-transformation of adenosine values (Figure S1B and S1C) followed by a univariable linear regression to identify potential associated variables (Table S4). Individual variables associated with a $P<0.2$ were identified including total number of vessels ($P=0.104$), age ($P=0.009$), hemoglobin A1c ($P=0.062$), sex ($P=0.193$), statin ($P=0.176$), P2Y12 (clopidogrel and ticagrelor) ($P=0.195$). After multivariable analysis (Table S5), only age ($P=0.039$) remained inversely associated with PAC.

Discussion

Despite abundant preclinical research linking adenosine to vascular disease, the current study is the first to evaluate the relationship of plasma adenosine levels with known cardiovascular risk factors, medical therapy and disease presence in humans. Herein, we report the performance of a high-throughput protocol for rapid HPLC-based adenosine quantification with performance parameters in congruence with good practice guidelines.[32] In the current cohort, our assay produces intra-subject and inter-subject variability consistent with other biomarkers of cardiovascular disease. Notably, in the current data set traditional cardiovascular risk factors and medical therapies were not associated with significant changes in plasma adenosine levels. In contrast, age and CAD presence were inversely associated with plasma adenosine levels—a finding for which age alone remained statistically significant after multivariable adjustment.

In our study of >1100 patients, traditional cardiovascular risk factors including hypertension, diabetes mellitus, family history, smoking, dyslipidemia, and sex did not associate with adenosine levels, while age was inversely correlated. Age is known to impact other established markers of cardiovascular disease. For example, low-density lipoprotein is known to diminish with advancing age at a rate of only 0.8% annually, though this still translates to important clinical implications.[36]

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Similarly, NT-proBNP (N-terminal pro-brain natriuretic peptide), an established marker for diagnosis, monitoring and outcomes in heart failure, is known to have a biological variance closely mirroring that of adenosine.[37,38] Moreover, it is also impacted by age, necessitating age-specific reference intervals and having diminished predictive abilities at more advanced ages.[39,40] Hence, while the annual incremental impact of advancing age on adenosine may be small, the cumulative impact of age over time remains an important consideration when establishing adenosine's performance as a diagnostic, prognostic and monitoring clinical test. Smokers have lower adenosine levels in their sputum, with increased adenosine levels and ADORA3 and 1 noted post cessation.[41] However, there has been no definitive link between smoking and circulating adenosine levels in keeping with our data. Similarly, extensive literature links diabetes mellitus to adenosine levels —however, these associations typically focus on augmented ADA levels, postulating that this leads to reduced circulating adenosine.[42] Our data do not demonstrate any overt differences in PAC between those with and without diabetes mellitus, while not evaluating an impact on receptor activity *nor* in specific vascular beds. Nonetheless, our study provides adequate power across subgroups to evaluate the impact of risk factors in humans and suggests that age may incrementally contribute to a decline in PAC—a finding which confounds smaller observational studies.

The use of medications for cardiovascular risk reduction did not demonstrate any significant associations with adenosine levels, with none of the angiotensin-converting enzyme inhibitors/ARBs, beta blockers, calcium channel blockers, statins, or heparins demonstrating any significant differences. In contrast, antiplatelet medications have been studied extensively for their putative impact on PAC. Specifically, ticagrelor has garnered significant attention with postulations that observed pleiotropic effects may stem from modulation of adenosine biology. In one study, 60 ACS patients were randomized to ticagrelor or clopidogrel with ticagrelor increasing plasma adenosine levels compared with those receiving clopidogrel, ostensibly via inhibition of red blood cell uptake.[17] However, a recent randomized crossover study in 54 ACS patients compared ticagrelor, prasugrel and clopidogrel—failed to demonstrate any significant augmentation in adenosine levels with ticagrelor compared with clopidogrel or prasugrel.[16] In our all-comers cohort with over 300 patients on ticagrelor therapy, reduced PAC was noted in those on ticagrelor compared with those not receiving ticagrelor. However, any non-randomized data set is innately confounded by the fact that ticagrelor is differentially employed in clinical practice, with ACS patients preferentially receiving ticagrelor given its superior clinical outcomes in ACS patients.[43] Indeed, this was observed in our data set with 68.8% of patients on ticagrelor presenting as an ACS versus only 27.9% of those not on ticagrelor. The differential use of ticagrelor in our cohort leads to innate differences between the populations which limit further analysis. Hence, our study was not intended to specifically address the impact of P2Y12 agents on adenosine levels but adds to the growing debate of the impact of ticagrelor on circulating adenosine levels.

Preclinical research has suggested adenosine plays an important role in modulating the pathogenesis of atherosclerosis particularly with modulation of systemic inflammation.[44–46] Indeed, our data suggest an inverse association between obstructive CAD and PAC. However, subgroup analysis failed to show any significant differences in adenosine levels across a spectrum of disease burden (ie, multivessel disease) or presentation (ie, acute coronary syndrome). Animal studies have noted increased activity of vascular ADA (resulting in reduced circulating adenosine levels) as a mediator of atherosclerosis—proposing ADA inhibition (augmenting circulating adenosine) as a possible therapeutic approach.[47] Similarly, genetic studies in humans lend support to the hypothesis that adenosine is a vascular protective molecule.[23–25] In humans, patients with CAD and genetic variations that purportedly augment circulating adenosine levels have reduced adverse cardiovascular events.[25] Our data now lend credence to this hypothesis—establishing a potential relationship between the presence and absence of disease. Whether adenosine acts as a prognosticator of events needs to be established in larger cohorts.

In spite of intensive research in the field of adenosine biology, the systematic development and evaluation of adenosine as a potential biomarker has not been previously performed owing largely to the technical limitations of sampling and existing quantification methods.[29] The currently reported assay yields technical performance that meets and exceeds good practice guidelines.[32] With a CV_a 3.2%, CV_i 35.8%, CV_g 56.7%, a RCV of 98.9%, and an II of 0.63, our assay performed in line with many known markers of coronary artery disease—such as C-reactive protein (CRP).[48–50] Indeed, from an assay perspective, a CV_a of 3.2% is markedly improved over early assays reporting CV_a ranging from 6% to 7%[18] and up to 10%,[19] while falling closely in line with contemporary assays yielding CV_a's of 1% to 3%, keeping with clinical assay standards.[20] The balance of these variances is crucial to assessing the clinical utility of a test. A test with high index of individuality (>1.4) will perform well as a diagnostic test based on population-level reference intervals, while a low index (<0.6) will not, as significant changes for a given subject may still fall within a population-based reference range.[29,31] Comparatively, CRP has a CV_a 5.2%, CV_i 42.2%, and CV_g 92.5%.[28] Having a large CV_g coupled with a relatively smaller CV_i means that individuals could have early disease-related changes without rising above a given reference interval, requiring relatively large changes in value before confidence in

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its significance is noted (Table S3).[27,28] Indeed, the RCV (smallest percentage change not likely because of CV_a or CV_i at significance of $P \leq 0.05$) is 118% for CRP and index of individuality was 0.46—a substantial change in value.[28] Comparatively, the RCV for adenosine in our assay is 98.9% with an II of 0.63, translating to similar considerations when determining its clinical utility and optimal interpretation. In fact, the variance of CRP leads to up to 46% of patients alternating between low- and high-risk categories despite a stable clinical status, translating to a 10% to 20% probability of making an erroneous risk assignment based on a single CRP value.[51] Despite this, CRP remains an established predictor of cardiovascular outcomes in those with[48] and without CAD[49] and predicts reduction of cardiovascular events in response to medical therapy[50]—supporting its role in current guidelines.[52,53] We demonstrate a similar variance profile to CRP for PAC in humans—meaning the variability in humans will require large sample sizes to adequately detect disease associations *or* to evaluate the impact of therapies on PAC.[54] Thus, clinical tests with this variance profile, such as adenosine, will have little utility in identifying early disease-related changes in the context of a healthy reference interval, favoring serial monitoring for significant changes in individual patients instead[28]—important implications for interpreting previous studies in humans and powering future evaluations of PAC as a marker or end point.

Certainly, our study is not without its limitations. The data are observational in nature and subject to all the limitations of this design. However, clinical and procedural data were prospectively collected in a nested registry design, limiting potential biases a solely retrospective approach may introduce. Second, the relatively large variability does open the possibility of regression dilution bias whereby significant differences may not be seen on account of inherent measurement errors.[55] Hence, despite being substantially larger than any previous human study, we are at risk of not detecting a modest association where one exists. Third, differences in absolute PAC values exist across varying collection methodologies reported. However, the uniform processing procedures and robust analytical variation of this study lends itself to unidirectional variance—whereby any potential errors would exist uniformly throughout the cohort and not impact the ability to detect biological differences present. Finally, our protocol was designed to evaluate adenosine levels assayed by peripheral collection - the primary method performed in humans. Hence, these values may not reflect levels in local tissues or specific vascular beds and thus does not preclude organ/tissue specific changes in adenosine. However, as demonstrated by our analysis, adequately powered studies to assess local adenosine levels may be difficult owing to technical factors and variability, with future studies requiring robust protocols and statistical methodology.

Conclusions

In humans, plasma adenosine levels are not significantly impacted by traditional cardiovascular risk factors or medical therapy for cardiovascular disease; however, advancing age and the presence of coronary artery disease may be associated with diminishing adenosine levels. Large prospective studies of basal levels and variation of adenosine for prediction of future cardiovascular events are warranted.

Sources of Funding

This work was supported by the University of Ottawa Heart Institute – Academic Medical Organization – Innovations Funding. Dr Beanlands is a Career Investigator supported by the Heart and Stroke Foundation of Ontario, a Tier 1 University of Ottawa Chair in Cardiovascular Research and Vered Chair in Cardiology.

Disclosures

None.

References

1. Daly JW. Adenosine receptors: targets for future drugs. *J Med Chem*. 1982;25:197 207.
2. Simard T, Jung R, Labinaz A, Faraz MA, Ramirez FD, Di Santo P, Pitcher I, Motazedian P, Gaudet C, Rochman R, Marbach J, Boland P, Sarathy K, Alghofaili S, Russo JJ, Couture E, Beanlands RS, Hibbert B. Adenosine as a marker and mediator of cardiovascular homeostasis: a translational perspective. *Cardiovasc Hematol Disord Drug Targets*. 2019;19.
3. Antonioli L, Blandizzi C, Csoka B, Pacher P, Hasko G. Adenosine signalling in diabetes mellitus pathophysiology and therapeutic considerations. *Nat Rev Endocrinol*. 2015;11:228 241.
4. Loffler M, Morote-Garcia JC, Eltzschig SA, Coe IR, Eltzschig HK. Physiological roles of vascular nucleoside transporters. *Arterioscler Thromb Vasc Biol*. 2007;27:1004 1013.
5. Yegutkin GG. Nucleotide- and nucleoside-converting ectoenzymes: important modulators of purinergic signalling cascade. *Biochim Biophys Acta*. 2008;1783:673 694.
6. Johnston-Cox HA, Koupenova M, Ravid K. A2 adenosine receptors and vascular pathologies. *Arterioscler Thromb Vasc Biol*. 2012;32:870 878.
7. Elliott MR, Chekeni FB, Trampont PC, Lazarowski ER, Kadl A, Walk SF, Park D, Woodson RI, Ostankovich M, Sharma P, Lysiak JJ, Harden TK, Leitinger N, Ravichandran KS. Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature*. 2009;461:282 286.
8. Feoktistov I, Goldstein AE, Ryzhov S, Zeng D, Belardinelli L, Voyno-Yasenetskaya T, Biaggioni I. Differential expression of adenosine receptors in human endothelial cells: role of A2B receptors in angiogenic factor regulation. *Circ Res*. 2002;90:531 538.
9. Pourdjabbar A, Hibbert B, Simard T, Ma X. Pathogenesis of neointima formation following vascular injury. *Cardiovasc Hematol Disord Drug Targets*. 2011;11:30 39.
10. Simard T, Hibbert B, Ramirez FD, Froeschl M, Chen YX, O'Brien ER. The evolution of coronary stents: a brief review. *Can J Cardiol*. 2014;30:35 45.
11. Yang D, Koupenova M, McCrann DJ, Kopeikina KJ, Kagan HM, Schreiber BM, Ravid K. The A2b adenosine receptor protects against vascular injury. *Proc Natl Acad Sci USA*. 2008;105:792 796.
12. Yang D, Zhang Y, Nguyen HG, Koupenova M, Chauhan AK, Makitalo M, Jones MR, Hilaire CS, Seldin DC, Toselli P, Lamperti E, Schreiber BM, Gavras H, Wagner DD, Ravid K. The A(2B) adenosine receptor protects against inflammation and excessive vascular adhesion. *J Clin Invest*. 2006;116:1913 1923.

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13. Chen J-F, Eltzschig HK, Fredholm BB. Adenosine receptors as drug targets what are the challenges? *Nat Rev Drug Discov*. 2013;12:265 286.
14. Kim H-H, Liao JK. Translational therapeutics of dipyridamole. *Arterioscler Thromb Vasc Biol*. 2008;28:s39 s42.
15. Al Jaroudi W, Iskandrian AE. Regadenoson: a new myocardial stress agent. *J Am Coll Cardiol*. 2009;54:1123 1130.
16. Ariotti S, Ortega-Paz L, van Leeuwen M, Brugaletta S, Leonardi S, Akkerhuis KM, Rimoldi SF, Janssens G, Gianni U, van den Berge JC, Karagiannis A, Windecker S, Valgimigli M. Effects of ticagrelor, prasugrel, or clopidogrel on endothelial function and other vascular biomarkers: a randomized crossover study. *JACC Cardiovasc Interv*. 2018;11:1576 1586.
17. Bonello L, Laine M, Kipson N, Mancini J, Helal O, Fromonot J, Gariboldi V, Condo J, Thuny F, Frere C, Camoin-Jau L, Paganelli F, Dignat-George F, Guieu R. Ticagrelor increases adenosine plasma concentration in patients with an acute coronary syndrome. *J Am Coll Cardiol*. 2014;63:872 877.
18. Huszar E, Barat E, Kollai M. Isocratic high-performance liquid chromatographic determination of plasma adenosine. *Chromatographia*. 1996;42:318 322.
19. Manfredi JP, Sparks HV Jr. Adenosine's role in coronary vasodilation induced by atrial pacing and norepinephrine. *Am J Physiol*. 1982;243:H536 H545.
20. Saadjian AY, Lévy S, Franceschi F, Zouher I, Paganelli F, Guieu RP. Role of endogenous adenosine as a modulator of syncope induced during tilt testing. *Circulation*. 2002;106:569 574.
21. Guieu R, Paganelli F, Sampieri F, Bechis G, Levy S, Rochat H. The use of HPLC to evaluate the variations of blood coronary adenosine levels during percutaneous transluminal angioplasty. *Clin Chim Acta*. 1994;230:63 68.
22. Funaya H, Kitakaze M, Node K, Minamino T, Komamura K, Hori M. Plasma adenosine levels increase in patients with chronic heart failure. *Circulation*. 1997;95:1363 1365.
23. Feldman AM, Wagner DR, McNamara DM. AMPD1 gene mutation in congestive heart failure: new insights into the pathobiology of disease progression. *Circulation*. 1999;99:1397 1399.
24. Loh E, Rebbeck TR, Mahoney PD, DeNofrio D, Swain JL, Holmes EW. Common variant in AMPD1 gene predicts improved clinical outcome in patients with heart failure. *Circulation*. 1999;99:1422 1425.
25. Anderson JL, Habashi J, Carlquist JF, Muhlestein JB, Horne BD, Bair TL, Pearson RR, Hart N. A common variant of the AMPD1 gene predicts improved cardiovascular survival in patients with coronary artery disease. *J Am Coll Cardiol*. 2000;36:1248 1252.
26. Andreassi MG, Botto N, Laghi-Pasini F, Manfredi S, Ghelarducci B, Farneti A, Solinas M, Biagini A, Picano E. AMPD1 (C34T) polymorphism and clinical outcomes in patients undergoing myocardial revascularization. *Int J Cardiol*. 2005;101:191 195.
27. Ricos C, Alvarez V, Cava F, Garcia-Lario JV, Hernandez A, Jimenez CV, Minchinela J, Perich C, Simon M. Current databases on biological variation: pros, cons and progress. *Scand J Clin Lab Invest*. 1999;59:491 500.
28. Macy EM, Hayes TE, Tracy RP. Variability in the measurement of C-reactive protein in healthy subjects: implications for reference intervals and epidemiological applications. *Clin Chem*. 1997;43:52 58.
29. Fraser CG, Harris EK. Generation and application of data on biological variation in clinical chemistry. *Crit Rev Clin Lab Sci*. 1989;27:409 437.
30. Jung RG, Simard T, Di Santo P, Labinaz A, Moreland R, Duchez AC, Majeed K, Motazedian P, Rochman R, Jung Y, Hibbert B. Performance of plasminogen activator inhibitor-1 as a biomarker in patients undergoing coronary angiography: analytical and biological considerations. *Diab Vasc Dis Res*. 2019. DOI: https://doi.org/10.1177/1479164119845123. [Epub ahead of print].
31. Meijers WC, van der Velde AR, Muller Kobold AC, Dijck-Brouwer J, Wu AH, Jaffe A, de Boer RA. Variability of biomarkers in patients with chronic heart failure and healthy controls. *Eur J Heart Fail*. 2017;19:357 365.
32. Shabir G. A practical approach to validation of HPLC methods under current good manufacturing practices. *J Valid Technol*. 2004;1:29 37.
33. Le May MR, So DY, Dionne R, Glover CA, Froeschl MP, Wells GA, Davies RF, Sherrard HL, Maloney J, Marquis JF, O'Brien ER, Trickett J, Poirier P, Ryan SC, Ha A, Joseph PG, Labinaz M. A citywide protocol for primary PCI in ST-segment elevation myocardial infarction. *N Engl J Med*. 2008;358:231 240.
34. Pizzi C, Xhyheri B, Costa GM, Faustino M, Flacco ME, Gualano MR, Fragassi G, Grigioni F, Manzoli L. Nonobstructive versus obstructive coronary artery disease in acute coronary syndrome: a meta-analysis. *J Am Heart Assoc*. 2016;5:e004185. DOI: 10.1161/JAHA.116.004185.
35. Hibbert B, MacDougall A, Labinaz M, O'Brien ER, So DY, Dick A, Glover C, Froeschl M, Marquis JF, Wells GA, Blondeau M, Le May MR. Bivalirudin for primary percutaneous coronary interventions: outcome assessment in the Ottawa STEMI registry. *Circ Cardiovasc Interv*. 2012;5:805 812.
36. Ferrara A, Barrett-Connor E, Shan J. Total, LDL, and HDL cholesterol decrease with age in older men and women. The Rancho Bernardo Study 1984 1994. *Circulation*. 1997;96:37 43.
37. Clerico A, Carlo Zucchelli G, Pilo A, Passino C, Emdin M. Clinical relevance of biological variation: the lesson of brain natriuretic peptide (BNP) and NT-proBNP assay. *Clin Chem Lab Med*. 2006;44:366 378.
38. Richards AM. Variability of NT-proBNP levels in heart failure: implications for clinical application. *Heart*. 2007;93:899 900.
39. Keyzer JM, Hoffmann JJ, Ringoir L, Nabbe KC, Widdershoven JW, Pop VJ. Age- and gender-specific brain natriuretic peptide (BNP) reference ranges in primary care. *Clin Chem Lab Med*. 2014;52:1341 1346.
40. Richards AM. The relationship of plasma NT-proBNP to age and outcomes in heart failure. *JACC Heart Fail*. 2016;4:746 748.
41. Versluis M, ten Hacken N, Postma D, Barroso B, Rutgers B, Geerlings M, Willemse B, Timens W, Hylkema M. Adenosine receptors in COPD and asymptomatic smokers: effects of smoking cessation. *Virchows Arch*. 2009;454:273 281.
42. Khemka VK, Bagchi D, Ghosh A, Sen O, Bir A, Chakrabarti S, Banerjee A. Raised serum adenosine deaminase level in nonobese type 2 diabetes mellitus. *ScientificWorldJournal*. 2013;2013:404320.
43. Wallentin L, Becker RC, Budaj A, Cannon CP, Emanuelsson H, Held C, Horrow J, Husted S, James S, Katus H, Mahaffey KW, Scirica BM, Skene A, Steg PG, Storey RF, Harrington RA, Freij A, Thorsen M. Ticagrelor versus clopidogrel in patients with acute coronary syndromes. *N Engl J Med*. 2009;361:1045 1057.
44. Barnholt KE, Kota RS, Aung HH, Rutledge JC. Adenosine blocks IFN-γ-induced phosphorylation of STAT1 on serine 727 to reduce macrophage activation. *J Immunol*. 2009;183:6767 6777.
45. Gessi S, Fogli E, Sacchetto V, Merighi S, Varani K, Preti D, Leung E, Maclennan S, Borea PA. Adenosine modulates HIF-1{alpha}, VEGF, IL-8, and foam cell formation in a human model of hypoxic foam cells. *Arterioscler Thromb Vasc Biol*. 2010;30:90 97.
46. Wang H, Zhang W, Zhu C, Bucher C, Blazar BR, Zhang C, Chen JF, Linden J, Wu C, Huo Y. Inactivation of the adenosine A2A receptor protects apolipoprotein E-deficient mice from atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2009;29:1046 1052.
47. Kutryb-Zajac B, Mateuszuk L, Zukowska P, Jasztal A, Zabielska MA, Toczek M, Jablonska P, Zakrzewska A, Sitek B, Rogowski J, Lango R, Slominska EM, Chlopicki S, Smolenski RT. Increased activity of vascular adenosine deaminase in atherosclerosis and therapeutic potential of its inhibition. *Cardiovasc Res*. 2016;112:590 605.
48. Liuzzo G, Biasucci LM, Gallimore JR, Grillo RL, Rebuzzi AG, Pepys MB, Maseri A. The prognostic value of C-reactive protein and serum amyloid a protein in severe unstable angina. *N Engl J Med*. 1994;331:417 424.
49. Pai JK, Pischon T, Ma J, Manson JE, Hankinson SE, Joshipura K, Curhan GC, Rifai N, Cannuscio CC, Stampfer MJ, Rimm EB. Inflammatory markers and the risk of coronary heart disease in men and women. *N Engl J Med*. 2004;351:2599 2610.
50. Ridker PM, MacFadyen JG, Everett BM, Libby P, Thuren T, Glynn RJ. Relationship of C-reactive protein reduction to cardiovascular event reduction following treatment with canakinumab: a secondary analysis from the CANTOS randomised controlled trial. *Lancet*. 2018;391:319 328.
51. Bogaty P, Dagenais GR, Joseph L, Boyer L, Leblanc A, Bélisle P, Brophy JM. Time variability of C-reactive protein: implications for clinical risk stratification. *PLoS One*. 2013;8:e60759.
52. Anderson TJ, Grégoire J, Pearson GJ, Barry AR, Couture P, Dawes M, Francis GA, Genest J Jr, Grover S, Gupta M, Hegele RA, Lau DC, Leiter LA, Lonn E, Mancini GBJ, McPherson R, Ngui D, Poirier P, Sievenpiper JL, Stone JA, Thanassoulis G, Ward R. 2016 Canadian Cardiovascular Society guidelines for the management of dyslipidemia for the prevention of cardiovascular disease in the adult. *Can J Cardiol*. 2016;32:1263 1282.
53. Stone NJ, Robinson JG, Lichtenstein AH, Bairey Merz CN, Blum CB, Eckel RH, Goldberg AC, Gordon D, Levy D, Lloyd-Jones DM, McBride P, Schwartz JS, Shero ST, Smith SC Jr, Watson K, Wilson PW. 2013 ACC/AHA guideline on the treatment of blood cholesterol to reduce atherosclerotic cardiovascular risk in adults: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *J Am Coll Cardiol*. 2014;63:2889 2934.
54. Platz EA, Sutcliffe S, De Marzo AM, Drake CG, Rifai N, Hsing AW, Hoque A, Neuhouser ML, Goodman PJ, Kristal AR. Intra-individual variation in serum C-reactive protein over four years: an implication for epidemiologic studies. *Cancer Causes Control*. 2010;21:847 851.
55. Hutcheon JA, Chiolero A, Hanley JA. Random measurement error and regression dilution bias. *BMJ*. 2010;340:c2289.

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Table S1. Repeatability assessment of adenosine assay.

	Injection	Retention Time	Peak Area	Peak Height
Standards	1	10.677	19635	1494
	2	10.665	19584	1460
	3	10.665	19210	1477
	4	10.656	19369	1473
	5	10.651	19194	1483
	6	10.65	18989	1477
	7	10.65	19029	1476
	8	10.643	18751	1469
	9	10.637	18403	1464
	10	10.638	18521	1471
	Mean	10.65	19068.50	1474.40
	SD	0.01	416.88	9.62
	CV (%)	0.12	2.19	0.65
	Injection	**Retention Time**	**Peak Area**	**Peak Height**
Samples	1	10.887	5906	384
	2	10.934	6088	374
	3	10.932	6024	391
	4	10.928	5861	384
	5	10.931	5906	389
	6	10.93	6036	396
	Mean	10.92	5970.17	386.33
	SD	0.02	90.85	7.55
	CV (%)	0.16	1.52	1.96

Generated via 10 sequential injections of 1000nM adenosine standard for standards assessment and 6 sequential injections of a single patient sample for samples assessment. Retention time in minutes. SD – standard deviation, CV – coefficient of variation

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Table S2. Linearity and range assessment of standards.

HPLC Parameters (N=3/level)		Retention Time (min)			Peak Area		
Adenosine (nM)	% Target	Mean	SD	CV (%)	Mean	SD	CV (%)
15000	**1500**	10.63	0.01	0.11	296199.00	2312.07	0.78
5000	**500**	10.65	0.01	0.05	98654.67	480.60	0.49
2000	**200**	10.68	0.01	0.09	39632.33	569.87	1.44
1000	**100**	10.66	0.02	0.16	19773.00	261.86	1.32
750	**75**	10.66	0.02	0.19	14807.67	371.25	2.51
500	**50**	10.68	0.00	0.02	9886.00	334.39	3.38
250	**25**	10.73	0.02	0.22	5397.33	379.43	7.03
100	**10**	10.75	0.01	0.07	2322.33	298.89	12.87

Generated via 3 separate injections of each concentration of adenosine standard ranging from 10-1500% of the target adenosine ceoncentration. HPLC – high performance liquid chromatography, SD – standard deviation, CV – coefficient of variation

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Table S3. Comparison of adenosine variation to established markers.

	CVi (%)	CVg (%)
Adenosine	**35.8**	**56.7**
C-reactive protein (CRP)	42.2-52.6	84.4-92.5
N-terminal (NT)-proBNP	30-50	99-130
Insulin	21.1	58.3
Vanillylmandelic acid (24 hour urine)	22.2	47
Cortisol	20.9	45.6
Creatinine kinase (CK)	22.8	40
Calcium (24 hour urine)	27.5	36.6
Haptoglobin	20.4	36.4
Lipase	23	33.1
Thyroid stimulating hormone (TSH)	19.7	27.2
Low-density lipoprotein (LDL)	8.3	25.7

CV – coefficient of variation, CVi – intra-subject CV, CVg – inter-subject CV[1-3]

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Table S4. Univariable linear regression.

	p	P<0.2
Age	0.009	*
Sex	0.193	*
Coronary artery disease - number of vessels	0.104	*
Acute coronary syndrome	0.950	
ST-elevation myocardial infarction	0.395	
Non-ST-elevation myocardial infarction	0.609	
Diabetes	0.219	
Smoking	0.998	
Dyslipidemia	0.298	
Family History	0.993	
Hypertension	0.553	
Prior coronary artery disease	0.536	
Prior myocardial infarction	0.243	
Prior percutaneous coronary intervention	0.387	
Prior coronary artery bypass grafting	0.734	
ACE inhibitor/Angiotensin receptor blocker	0.483	
Acetylsalicylic acid	0.864	
Beta Blocker	0.422	
Calcium Channel Blocker	0.379	
Statin	0.176	*
Intravenous unfractionated heparin	0.558	
Subcutaneous low molecular weight heparin	0.516	
P2Y12	0.195	*
Hemoglobin A1c	0.062	*
Creatinine	0.816	
Hemoglobin	0.921	

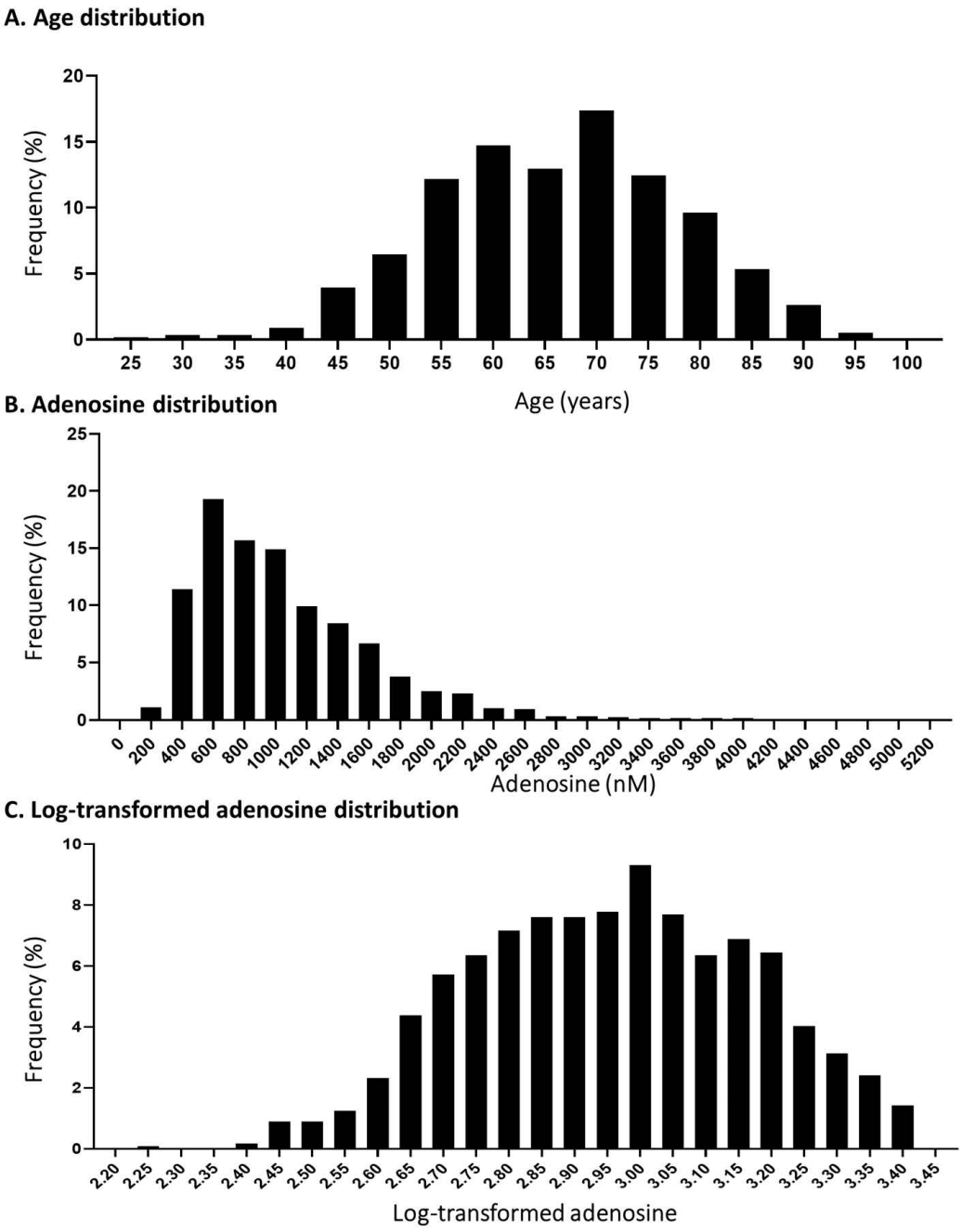
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Table S5. Multivariable linear regression.

	p	p<0.05
Age	0.039	*
Sex	0.237	
Clopidogrel	0.297	
Ticagrelor	0.537	
Statin	0.504	
Coronary artery disease - number of vessels	0.332	
Hemoglobin A1c	0.088	

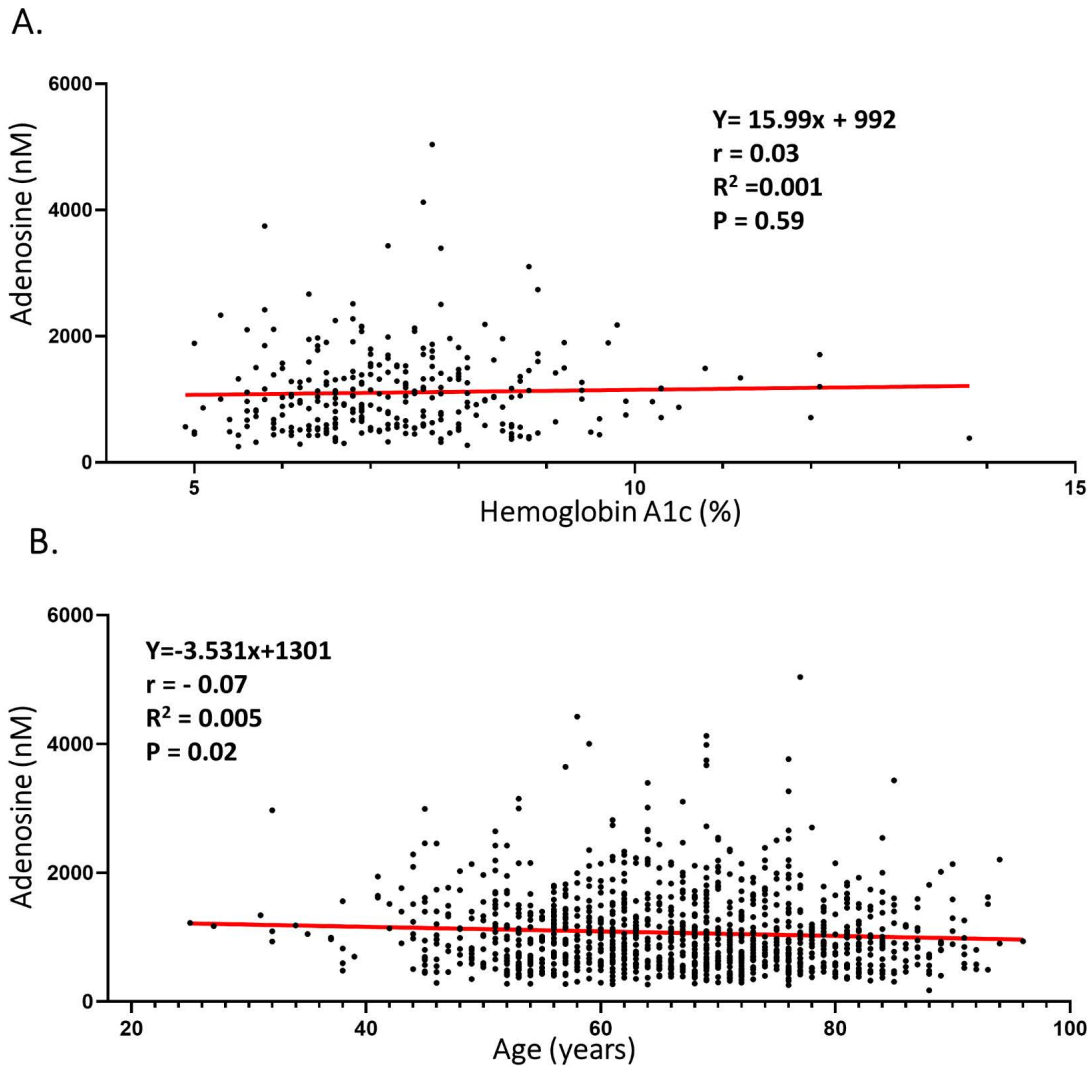
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Figure S1. Distribution of adenosine and age. Histograms depicting the relative frequency in percentage (%) for each designated bin of term. Specifically, (A) demonstrates age distribution throughout the entire cohort (N=1,141) of patients undergoing workup for coronary artery disease, while (B) provides adenosine distribution across the entire cohort. **(C)** Logarithmic transformation of adenosine values across the entire cohort.



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Figure S2. Association of hemoglobin A1c and age with adenosine. (A) Univariable analysis completed for all diabetic patients with a hemoglobin A1c indexed (N =294), demonstrating no significant relationship between HbA1c and adenosine (p=0.59). **(B)** Univariable linear regression of adenosine levels and age (years) in the entire cohort (N= 1,141) demonstrating a negative correlation (p=0.02).



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Supplemental References:

1. Ricos C, Alvarez V, Cava F, Garcia-Lario JV, Hernandez A, Jimenez CV, Minchinela J, Perich C, Simon M. Current databases on biological variation: Pros, cons and progress. *Scandinavian journal of clinical and laboratory investigation*. 1999;59:491-500.

2. Macy EM, Hayes TE, Tracy RP. Variability in the measurement of c-reactive protein in healthy subjects: Implications for reference intervals and epidemiological applications. *Clinical Chemistry*. 1997;43:52-58.

3. Clerico A, Carlo Zucchelli G, Pilo A, Passino C, Emdin M. Clinical relevance of biological variation: The lesson of brain natriuretic peptide (bnp) and nt-probnp assay. *Clin Chem Lab Med*. 2006;44:366-378.

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Appendix L

Journal initiatives to enhance preclinical research: analyses of stroke, nature medicine, science translational medicine

Citation information:

Ramirez FD, **Jung RG**, Motazedian P, Perry-Nguyen D, Di Santo P, MacDonald Z, Clancy A, Labinaz A, Promislow S, Simard T, Provencher S, Bonnet S, Graham I, Wells G, Hibbert B. Journal initiatives to enhance preclinical research: Analyses of Stroke, Nature Medicine, Science Translational Medicine. **(2020)**. *Stroke.*

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Journal Initiatives to Enhance Preclinical Research: Analyses of Stroke, Nature Medicine, Science Translational Medicine

Author: F. Daniel Ramirez, Richard G. Jung, Pouya Motazedian, et al

Publication: Stroke

Publisher: Wolters Kluwer Health, Inc.

Date: Nov 13, 2019

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Journal Initiatives to Enhance Preclinical Research

Analyses of *Stroke, Nature Medicine, Science Translational Medicine*

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Background and Purpose—Preclinical research using animals often informs clinical trials. However, its value is dependent on its scientific validity and reproducibility, which are, in turn, dependent on rigorous study design and reporting. In 2011, *Stroke* introduced a Basic Science Checklist to enhance the reporting and methodology of its preclinical studies. Except for *Nature* and *Science* journals, few others have implemented similar initiatives. We sought to estimate the impact of these journal interventions on the quality of their published reports.

Methods—All articles published in *Stroke*, *Nature Medicine*, and *Science Translational Medicine* over 9 to 18 years and in 2 control journals without analogous interventions over a corresponding 11.5 years were reviewed to identify reports of experiments in nonhuman mammals with proposed clinical relevance. The effect of journal interventions on the reporting and use of key study design elements was estimated via interrupted time-series analyses.

Results—Of 33 009 articles screened, 4162 studies met inclusion criteria. In the 3.5 to 12 years preceding each journal's intervention, the proportions of studies reporting and using key study design elements were stable except for blinding in *Stroke* and randomization in *Science Translational Medicine*, which were both increasing. Post-intervention, abrupt and often marked increases were seen in the reporting of randomization status (level change: +17% to +44%, $P \leq 0.005$), blinding (level change: +20% to +40%, $P \leq 0.008$), and sample size estimation (level change: 0% to +40%, $P \leq 0.002$ in 2 journals). Significant but more modest improvements in the use of these study design elements were also observed. These improvements were not seen in control journals.

Conclusions—Journal interventions such as *Stroke*'s author submission checklist can meaningfully improve the quality of published preclinical research and should be considered to enhance study transparency and design. However, such interventions are alone insufficient to fully address widespread shortcomings in preclinical research practices.

Visual Overview—An online visual overview is available for this article. **(*Stroke*. 2020;51:291-299. DOI: 10.1161/STROKEAHA.119.026564.)**

Key Words: animal model ■ bias ■ biomedical research ■ research design ■ sample size

See related article, p 6

Preclinical experiments using animal models are an integral part of biomedical research. However, their value is dependent on their scientific validity and reproducibility,[1,2] which are, in turn, dependent on rigorous study design and reporting.[3,4] Poorly designed or inadequately reported preclinical studies can, therefore, reduce the dividends from research investments,[5,6] result in animals being unjustifiably subjected to harm,[2,7,8] and inappropriately spur or deter clinical trials in humans.[3,9,10] Randomization, blinding, sample size estimation,

Received June 2, 2019; final revision received August 8, 2019; accepted August 28, 2019.

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The online-only Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/STROKEAHA.119.026564.

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and considering sex as a biological variable are considered essential study design elements (SDEs) to improve the reproducibility and predictive value of preclinical experiments.[1,8,11–14] Randomization protects against selection bias; blinding protects against performance, detection, and attrition biases; sample size estimation ensures adequate statistical power, which improves effect estimate precision and protects against spurious conclusions; and considering sex as a biological variable increases the likelihood that results will be relevant to both men and women.[2,5,7,14–16] These SDEs are routinely used in clinical trials but are rarely implemented at the preclinical stage, even though animal and human studies are susceptible to similar risks of bias and threats to study validity.[8,12,14,15]

Recently, the National Institutes of Health and prominent journals, including *Nature* and *Science* publishing groups, have focused considerable effort on improving the rigor and reproducibility of preclinical research, particularly through enhanced experimental conduct and reporting standards.[1,11,13,17] In May 2013, *Nature* journals introduced editorial measures that included a mandatory checklist to prompt authors to disclose information regarding crucial experimental SDEs[18]—a similar initiative to the Basic Science Checklist introduced by *Stroke* in 2011.[19] *Science Translational Medicine* (*Science TM*) similarly began requiring that authors describe and defend their study designs, including disclosing whether key SDEs were implemented.[20] Yet, robust data on the impact of such interventions are limited and at times contradictory.[12,19,21–23] We hypothesized that the introduction of targeted journal initiatives had improved the reporting and experimental design of published preclinical research.

Methods

The data that support the findings of this study are available from the corresponding author on reasonable request.

Study Screening and Selection

All studies published in *Stroke* between January 2000 and December 2017 were reviewed. The start date of January 2000 was selected as the first Stroke Academic Industry Roundtable guidelines on preclinical experiments were published exclusively in *Stroke* in December 1999,[24] arguably marking an editorial awareness of their goals and importance. The screening and study selection process for this journal has been described previously.[12] Briefly, studies were included if they described original research published as full articles; reported results of in vivo experiments in nonhuman mammals; and described pathophysiology, genetics, or therapeutic interventions that were stated to be directly relevant to a specific disorder in humans. Studies on physiological or genetic characteristics were included if potential therapeutic applications or implications were proposed in the article. These criteria were used in an effort to enrich our study sample with confirmatory or proof-of-concept experiments[2,25,26] while minimizing the inclusion of reports of exploratory experiments with as yet unclear relevance to human disorders. All articles in *Stroke* were independently selected and data extracted using standardized case report forms by 2 reviewers, including via review of all articles' supplementary material. Discrepancies were resolved by consensus or by an independent adjudicator. Our group has reported 94.5% interrater agreement for study inclusion before resolution using this approach in a previous review of >28 000 articles.[12]

All studies published in *Nature Medicine* and *Science TM* between 2009 and 2017 were similarly reviewed, but the study selection process differed slightly for these journals as described in methods in the online-only Data Supplement. Data extraction was performed by 2 independent assessors as was done for *Stroke*. The starting year of 2009 for data collection was selected because the inaugural issue of *Science TM* was published in that year.

We previously examined the reported methodological quality of articles published in an additional 4 leading cardiovascular journals between July 2006 and June 2016 using before-after comparisons.[12] Two of these journals (*Circulation* and *Circulation Research*) publish on all aspects of cardiovascular medicine, allocate a substantial amount of space to preclinical research that is of similar reported methodological quality,[12] have among the highest journal impact factors in the cardiovascular field, and did not explicitly and publicly implement initiatives to improve the reporting or methodology of their articles until October 2017.[17] Limited journal- and disease-specific data from those before-after comparisons have been reported previously.[12,14,27] The data set from these journals was extended to December 2017, expanded in scope (see below), and combined into a single control group to increase statistical power. Study selection and data extraction for these control journals were performed as was done for *Stroke*.

Diseases or disease systems of interest in each journal are listed in methods in the online-only Data Supplement. Studies on sex-specific diseases (for instance, prostate or ovarian cancer) were prospectively identified during data collection.

Data Extraction

The date of publication, disease system studied, and animal model(s) used were collected for all articles. Disease systems studied and animal models used in <2% of reports in all journals were included in "other" categories. Data on randomization, blinding (concealed allocation or blinded outcome assessment), sample size/power estimations, and the sex of the animals used were also collected. These data have been identified as minimum reporting requirements by the National Institutes of Health.[2,13] A distinction was made between the reporting of any given SDE and its reported use such that cases in which studies reported not randomizing animals to treatment groups or *not* using blinding, for instance, were captured. Similarly, studies that reported using a specific SDE, but its description revealed that it had not been properly executed, were captured (eg, studies that reported being adequately powered because group comparisons yielded statistically significant results). The reporting and use of SDEs are, therefore, described separately. These elements were deemed to have been reported on or used if done for any experiment or animal model within an article. Because of the small number of *Science TM* studies published in 2009 (the first issue was published in October of that year), studies from 2009 to 2010 were combined for that journal for all analyses. Similarly, studies published in control journals between July and December 2006 were included with articles published in 2007.

Given the volume of articles screened, journal-specific stylistic features (page layouts, article organization, color schemes, and appearance of figures), and the variability and volume of supplementary materials, de-identification of articles was not performed and reviewers were not blinded to journal or date of publication during screening or data extraction, although they were blinded to scores from other reviewers. Articles were also reviewed in a predominantly chronological sequence rather than in a randomized fashion.

Statistical Analysis

Journal-specific sample size calculations are described in methods in the online-only Data Supplement. Categorical variables are reported as number (%) and were compared via χ^2 tests. Continuous variables are reported as mean±SD and were compared using *t* tests. Interrater agreement for study inclusion for *Stroke* and control journals was calculated using percent agreement and Cohen's κ statistic. Secular trends in the number of preclinical studies published over time were examined using Spearman rank correlation coefficients (ρ); trends in the proportions of studies reporting on or using SDEs were examined using Cochrane-Armitage trend tests. Randomization, blinding, and sample size estimation were specifically targeted in all initiatives studied; therefore, temporal changes in the proportions of studies

reporting on and using these SDEs were evaluated as journal-specific interrupted time series with editorial initiatives introduced as step functions in segmented regression models and are reported as absolute cumulative means or absolute percentages with 95% CIs. Data on sex reporting and sex of animals used were similarly but separately evaluated and excluded studies on sex-specific disorders. For temporal analyses, *Stroke* was examined in 12-month intervals whereas *Nature Medicine*, *Science TM*, and control journals were examined in 6-month intervals to render sample sizes similar per time point between journals. The influence of journal initiatives was also estimated by comparing reporting and methodological quality measures before versus after their implementation in each journal and via simple and multivariable modified Poisson regression models with robust error variance (sandwich estimation) to generate journal-specific crude and adjusted relative risks (RRs) with 95% CIs. Covariate category inclusion in multivariable models was prespecified and included relevant disease system studied and animal model used. Within these covariate categories, specific disease system and animal model covariates were selected via backward elimination using an inclusion criterion of $P<0.20$ to ensure adequate events per predictor variable. All analyses were performed using SAS 9.4 (SAS Institute Inc, Cary, NC) using a 2-tailed α level of 0.05 to define statistical significance.

Results

Study Selection and Characteristics

Of 33 009 indexed articles, 4162 met inclusion criteria and were analyzed (Figure I in the online-only Data Supplement). Interrater agreement for study inclusion before resolution was 97.9% (κ=0.82). The number of relevant articles published yearly in *Nature Medicine* and in control journals remained stable over the period examined (105±10, ρ=0.234, P=0.544 and 118±20, ρ=−0.378, P=0.252), but increased and decreased in *Science TM* (108±28, ρ=0.976, $P<0.001$), and *Stroke* (55±13, ρ=−0.634, P=0.005), respectively (Figure II in the online-only Data Supplement).

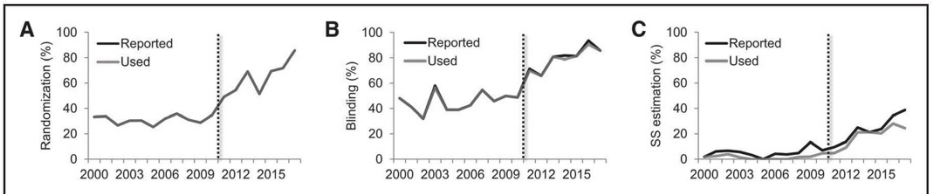
In *Stroke* and in control journals, ≥93.0% of studies focused on cardiovascular diseases and ≥85.5% used mice or rats. In *Nature Medicine* and *Science TM*, the most commonly studied disease systems were oncology, infectious diseases, neurology, endocrinology, cardiovascular, and "other," with 71.3% and 85.6% using mice in each journal, respectively. Psychiatry, anesthesia, obstetrics/gynecology, ophthalmology, and toxicology were each studied in <2% of studies in all journals and were therefore included in the "other" category (Table I in the online-only Data Supplement). One hundred eight articles were deemed to be sex-specific and were excluded from sex-based analyses.

Stroke

In the 12 years leading up to and including the year in which *Stroke* introduced its Basic Science Checklist, the proportions of studies that reported on and used blinding were increasing (P_{trend}=0.002 for both) whereas the reporting or use of randomization and sample size estimations were stable. Beginning in 2012, significant trend level or slope increases in the reporting and use of these SDEs were observed (Figure 1). No study reported not having randomized animals, and 2 studies reported not having used blinding after the checklist was introduced (Table). Among studies that reported having randomized animals, the proportion describing the method of randomization increased from 14/233 (6.0%) to 30/183 (16.4%; $P<0.001$). Temporal increases in the cumulative numbers of reported and used SDEs were observed (Figure III in the online-only Data Supplement).

Nature Medicine

In the 4.5 years preceding *Nature Medicine*'s author submission checklist, the reporting of randomization, blinding status, or sample size estimation remained stable. After the journal implemented its checklist, significant improvements in the trend lines for the reporting and use of randomization, blinding, and sample size estimation were observed. Large numbers of articles reported not having implemented various SDEs as shown by diverging trend lines of the reporting and use of individual SDEs after the initiative (Figure 2) and as shown in the Table. Among studies that reportedly randomized animals, the proportion describing the method used for randomization did not appreciably change following checklist implementation (1/50 [2.0%] versus 14/245 [5.7%], P=0.480). Corresponding temporal increases in the cumulative number



Panel	SDE	Δ Level (95% CI)	P	Δ Slope (95% CI)	P
A	Randomization (reported)	+17% (6, 28)	0.005	+5% (3, 8)	<0.001
	Randomization (used)	+17% (6, 28)	0.005	+5% (3, 8)	<0.001
B	Blinding (reported)	+20% (6, 34)	0.008	+3% (0, 6)	0.073
	Blinding (used)	+19% (6, 32)	0.008	+3% (0, 6)	0.063
C	SS estimation (reported)	+2% (-5, 9)	0.488	+4% (3, 6)	<0.001
	SS estimation (used)	+6% (1, 12)	0.034	+3% (2, 5)	<0.001

Figure 1. Reporting and use of individual study design elements (SDEs) in *Stroke*. **A**, Randomization, (**B**) blinding, and (**C**) sample size (SS) estimation. Vertical interrupted line denotes the journal intervention. Δ Level/Slope indicates change in level/slope.

Table. Comparison of Study Design Element Reporting and Use in Preclinical Studies Before and After the Implementation of Relevant Initiatives in *Stroke*, *Nature Medicine*, *Science Translational Medicine*, and Control Journals

	Before, n (%)	After, n (%)	Crude RR, (95% CI)	*P* Value	Adjusted RR, (95% CI)*	*P* Value*
Stroke	N=715	N=269				
Study design element reporting						
Randomization	233 (32.6)	183 (68.0)	2.1 (1.8–2.4)	<0.0001	2.2 (2.0–2.6)	<0.0001
Blinding	338 (47.3)	218 (81.0)	1.7 (1.6–1.9)	<0.0001	1.7 (1.6–1.9)	<0.0001
Sample size estimation	40 (5.6)	70 (26.0)	4.7 (3.2–6.7)	<0.0001	5.5 (3.8–8.1)	<0.0001
Sex of animals used	601 (84.1)	246 (91.5)	1.1 (1.0–1.1)	<0.001	1.1 (1.0–1.1)	0.008
Study design element use						
Randomization	233 (32.6)	183 (68.0)	2.1 (1.8–2.4)	<0.0001	2.2 (2.0–2.6)	<0.0001
Blinding	336 (47.0)	216 (80.3)	1.7 (1.5–1.9)	<0.0001	1.7 (1.6–1.9)	<0.0001
Sample size estimation	14 (2.0)	55 (20.5)	10.4 (5.9–18.5)	<0.0001	14.1 (7.8–25.5)	<0.0001
Inclusion of both sexes	50 (7.0)	26 (9.7)	1.1 (1.0–1.2)	0.044	1.1 (1.0–1.2)	0.090
Nature Medicine	N=476	N=473				
Study design element reporting						
Randomization	50 (10.5)	356 (75.3)	7.2 (5.5–9.4)	<0.0001	7.1 (5.4–9.3)	<0.0001
Blinding	112 (23.5)	390 (82.5)	3.5 (3.0–4.1)	<0.0001	3.5 (3.0–4.1)	<0.0001
Sample size estimation	6 (1.3)	334 (70.6)	56.0 (25.2–124.3)	<0.0001	NR	
Sex of animals used	311 (68.5)†	414 (91.2)†	1.3 (1.2–1.4)	<0.0001	1.3 (1.2–1.4)	<0.0001
Study design element use						
Randomization	50 (10.5)	245 (51.8)	4.9 (3.7–6.5)	<0.0001	4.8 (3.7–6.4)	<0.0001
Blinding	112 (23.5)	226 (47.8)	2.0 (1.7–2.4)	<0.0001	2.0 (1.7–2.4)	<0.0001
Sample size estimation	1 (0.2)	145 (30.7)	145.9 (20.5–1038.6)	<0.0001	NR	
Inclusion of both sexes	98 (21.6)†	186 (41.0)†	1.5 (1.4–1.6)	<0.0001	1.5 (1.3–1.6)	<0.0001
Science Translational Medicine	N=290	N=574				
Study design element reporting						
Randomization	54 (18.6)	375 (65.3)	3.5 (2.7–4.5)	<0.0001	3.5 (2.7–4.4)	<0.0001
Blinding	88 (30.3)	404 (70.4)	2.3 (1.9–2.8)	<0.0001	2.3 (2.0–2.8)	<0.0001
Sample size estimation	14 (4.8)	274 (47.7)	9.9 (5.9–16.6)	<0.0001	NR	
Sex of animals used	175 (64.8)‡	370 (69.0)‡	1.1 (1.0–1.2)	0.238	1.1 (1.0–1.2)	0.284
Study design element use						
Randomization	53 (18.3)	335 (58.4)	3.2 (2.5–4.1)	<0.0001	3.2 (2.5–4.1)	<0.0001
Blinding	85 (29.3)	280 (48.8)	1.7 (1.4–2.0)	<0.0001	1.7 (1.4–2.1)	<0.0001
Sample size estimation	4 (1.4)	146 (25.4)	18.4 (6.9–49.3)	<0.0001	NR	
Inclusion of both sexes	47 (17.4)‡	121 (22.6)‡	1.2 (1.0–1.3)	0.022	1.1 (1.0–1.3)	0.035
Control journals	*N*=889§	*N*=476§				
Study design element reporting						
Randomization	169 (19.0)	93 (19.5)	1.0 (0.8–1.3)	0.813	1.1 (0.8–1.3)	0.614
Blinding	308 (34.7)	161 (33.8)	1.0 (0.8–1.1)	0.761	1.0 (0.8–1.1)	0.839
Sample size estimation	31 (3.5)	42 (8.8)	2.5 (1.6–4.0)	<0.0001	2.7 (1.7–4.1)	<0.0001
Sex of animals used	626 (70.9)‖	361 (76.3)‖	1.1 (1.0–1.1)	0.028	1.1 (1.0–1.1)	0.041
Study design element use						
Randomization	164 (18.5)	91 (19.1)	1.0 (0.8–1.3)	0.762	1.1 (0.9–1.3)	0.575

(*Continued*)

Table. Continued

	Before, n (%)	After, n (%)	Crude RR, (95% CI)	*P* Value	Adjusted RR, (95% CI)*	*P* Value*
Blinding	307 (34.5)	159 (33.4)	1.0 (0.8–1.1)	0.676	1.0 (0.8–1.1)	0.765
Sample size estimation	8 (0.9)	20 (4.2)	4.7 (2.1–10.5)	<0.001	NR	
Inclusion of both sexes	143 (16.2)‖	76 (16.1)‖	1.0 (0.8–1.3)	0.952	0.9 (0.7–1.2)	0.613

NR indicates not reported due to small number of events per predictor variable; and RR, relative risk.
*Adjusted for most relevant disease system studied and animal model used.
†N=454 and 454 before and after, respectively (excluding studies on sex-specific diseases).
‡N=270 and 536 before and after, respectively (excluding studies on sex-specific diseases).
§Before and after the introduction *Nature Medicine*/*Science TM*'s initiatives. All effect estimates were similar when analyzed as before versus after the introduction of *Stroke*'s initiative (data not shown).
‖N=883 and 473 before and after, respectively (excluding studies on sex-specific diseases).

of reported and used SDEs per article were detected (Figure III in the online-only Data Supplement).

Science Translational Medicine

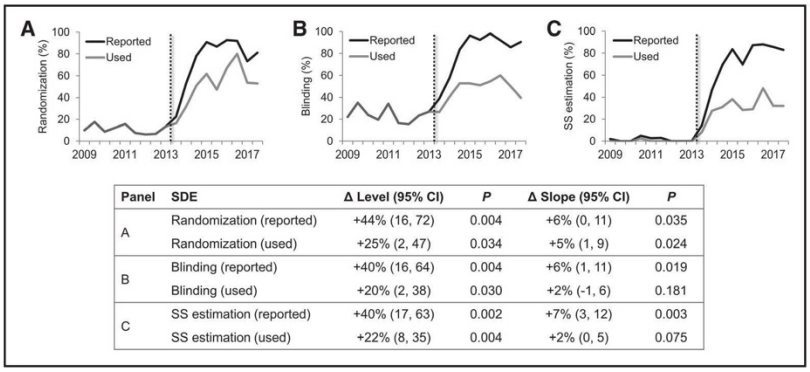
In the 3.5 years before *Science TM* revised its reporting requirements and policies, both the reporting and use of randomization were increasing (P_{trend}=0.004 and 0.007, respectively) whereas the reporting and use of blinding and sample size estimation were stable. After the introduction of this journal's initiative, significant improvements were also observed in the reporting of randomization, blinding status, and sample size estimations and in the use of randomization and sample size estimations. As was seen in *Nature Medicine*, a substantial number of studies reported not having used specific SDEs, particularly blinding and power calculations (Figure 3, Table), but among studies that reported using randomization there was no change in the proportion describing how it was performed (0/53 [0%] versus 16/335 [4.8%], *P*=0.143). Temporal increases in the cumulative numbers of reported and used SDEs were also detected (Figure III in the online-only Data Supplement).

Control Journals

In contrast, in control journals, there were no temporal improvements in the reporting or use of randomization, blinding, or sample size calculations over a corresponding 11.5 year period (Figure 4), excepting a modest improvement in the reporting of sample size calculations beginning in 2012 when analyzed using *Stroke*'s initiative as the time series interruption (1% absolute trend line slope increase [95% CI, 0%–2%], *P*=0.049; data not shown). No changes in the cumulative numbers of reported or implemented SDEs were detected in these control journals (Figure III in the online-only Data Supplement).

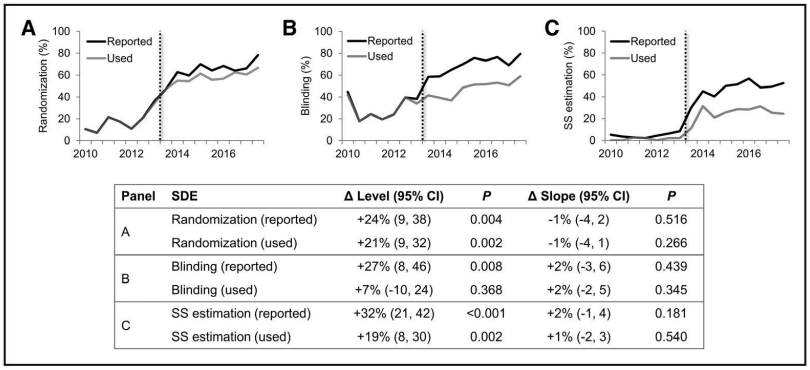
Sex Reporting and Inclusion

The proportions of articles published in *Stroke* that reported the sex of the animals used and included both males and females in experiments were stable before its checklist was introduced and did not improve afterwards. The proportions of studies in *Nature Medicine* and *Science TM* that reported the sex of the animals used and included animals of both sexes were increasing before their respective interventions (P_{trend} ≤0.040 for both in both journals), but also did not improve after the journals' initiatives. In control journals, before *Nature Medicine*, *Science TM*, or *Stroke*'s interventions, the proportions of articles reporting the sex of animals used were stable and the proportions reporting the use of both males and females were decreasing (P_{trend} <0.001). A trend level increase (+10%, [95% CI, 0%–20%] *P*=0.050) and slope increase (+2%, [95% CI, 0%–3%] *P*=0.014) in the use of both sexes were observed in these journals when analyzed using *Stroke*'s initiative as the time series interruption, but not when analyzed using *Nature Medicine* or *Science TM*'s initiatives. A marked and persistent preferential use of male animals was evident in articles in *Stroke* and control journals (Figure 5).



Panel	SDE	Δ Level (95% CI)	*P*	Δ Slope (95% CI)	*P*
A	Randomization (reported)	+44% (16, 72)	0.004	+6% (0, 11)	0.035
	Randomization (used)	+25% (2, 47)	0.034	+5% (1, 9)	0.024
B	Blinding (reported)	+40% (16, 64)	0.004	+6% (1, 11)	0.019
	Blinding (used)	+20% (2, 38)	0.030	+2% (-1, 6)	0.181
C	SS estimation (reported)	+40% (17, 63)	0.002	+7% (3, 12)	0.003
	SS estimation (used)	+22% (8, 35)	0.004	+2% (0, 5)	0.075

Figure 2. Reporting and use of individual study design elements (SDEs) in *Nature Medicine*. **A**, Randomization, (**B**) blinding, and (**C**) sample size (SS) estimation. Vertical interrupted line denotes the journal intervention. Δ Level/Slope indicates change in level/slope.



Panel	SDE	Δ Level (95% CI)	*P*	Δ Slope (95% CI)	*P*
A	Randomization (reported)	+24% (9, 38)	0.004	-1% (-4, 2)	0.516
	Randomization (used)	+21% (9, 32)	0.002	-1% (-4, 1)	0.266
B	Blinding (reported)	+27% (8, 46)	0.008	+2% (-3, 6)	0.439
	Blinding (used)	+7% (-10, 24)	0.368	+2% (-2, 5)	0.345
C	SS estimation (reported)	+32% (21, 42)	<0.001	+2% (-1, 4)	0.181
	SS estimation (used)	+19% (8, 30)	0.002	+1% (-2, 3)	0.540

Figure 3. Reporting and use of individual study design elements (SDEs) in *Science Translational Medicine*. **A**, Randomization, (**B**) blinding, and (**C**) sample size (SS) estimation. Vertical interrupted line denotes the journal intervention. Δ Level/Slope indicates change in level/slope.

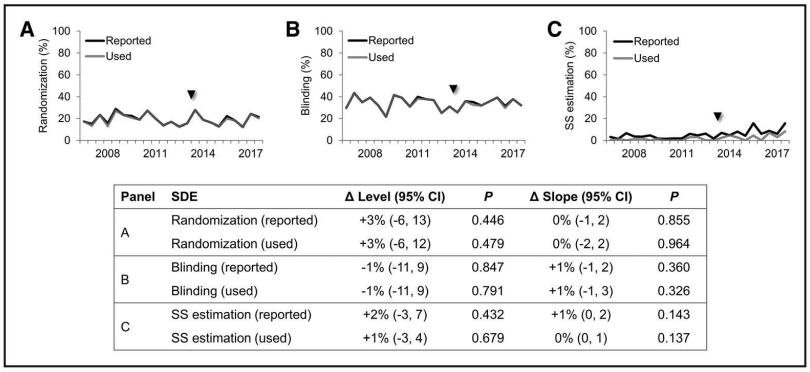
Adjusted Analyses

All temporal patterns persisted in before-after comparisons adjusting for the most relevant disease studied and animal model(s) used (Table).

Discussion

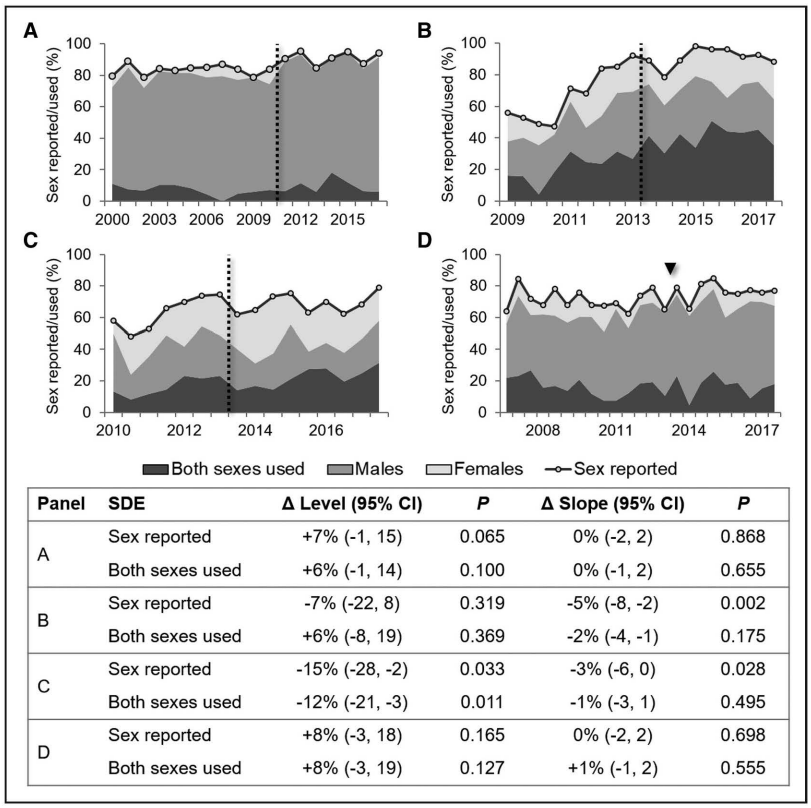
Time-series analyses identified significant and marked improvements in the quality of reporting of published preclinical studies coinciding with the introduction of relevant initiatives in three journals—findings that persisted after accounting for secular trends in reporting and methodological practices and that were not seen in influential journals without analogous interventions over the same period. These findings strongly support the notion that journal interventions can meaningfully influence the quality of published preclinical research.

Mechanistically, well-designed author submission checklists and analogous journal initiatives could improve the quality of published preclinical research in at least 4 ways: (1) by improving the reporting of study methods without affecting the methodology used (ie, improving transparency for readers)[28]; (2) by improving transparency for journal editors and peer reviewers, which could influence whether a manuscript is accepted for publication[29]; (3) by influencing authors' decision to submit to a particular journal (eg, dissuading authors from submitting a manuscript if their study is felt to have methodological shortcomings that will be highlighted)[17]; and (4) by influencing experimental study design practices among researchers who hope to publish in a particular journal.[28] However, few robust analyses of the impact of journal initiatives on published research quality exist. The NPQIP (Nature Publication Quality Improvement Project) Collaborative group examined the potential impact of *Nature* journals' checklist on the reporting quality of in vivo research in 448 articles using primarily a controlled before-after study design.[21] Our analysis of *Nature* journals' publications differs by focusing on *Nature Medicine* (considered to be focused on translational research) and by evaluating a larger sample size (n=949) over a longer period to increase our confidence that changes observed were due to the publishing group's intervention rather than secular trends in editorial practices or culture. Nevertheless, our data support their conclusions and expand on them by demonstrating improvements in study quality after instituting reporting and methodological standards at other journals. The recently reported IICARus



Panel	SDE	Δ Level (95% CI)	*P*	Δ Slope (95% CI)	*P*
A	Randomization (reported)	+3% (-6, 13)	0.446	0% (-1, 2)	0.855
	Randomization (used)	+3% (-6, 12)	0.479	0% (-2, 2)	0.964
B	Blinding (reported)	-1% (-11, 9)	0.847	+1% (-1, 2)	0.360
	Blinding (used)	-1% (-11, 9)	0.791	+1% (-1, 3)	0.326
C	SS estimation (reported)	+2% (-3, 7)	0.432	+1% (0, 2)	0.143
	SS estimation (used)	+1% (-3, 4)	0.679	0% (0, 1)	0.137

Figure 4. Reporting and use of individual study design elements (SDEs) in control journals. **A**, Randomization, (**B**) blinding, and (**C**) sample size (SS) estimation. Analyzed using *Nature Medicine* and *Science TM*'s initiatives as the time series interruption (arrowhead; similar results were obtained using *Stroke*'s initiative as interruption). Δ Level/Slope indicates change in level/slope.



Panel	SDE	Δ Level (95% CI)	*P*	Δ Slope (95% CI)	*P*
A	Sex reported	+7% (-1, 15)	0.065	0% (-2, 2)	0.868
	Both sexes used	+6% (-1, 14)	0.100	0% (-1, 2)	0.655
B	Sex reported	-7% (-22, 8)	0.319	-5% (-8, -2)	0.002
	Both sexes used	+6% (-8, 19)	0.369	-2% (-4, -1)	0.175
C	Sex reported	-15% (-28, -2)	0.033	-3% (-6, 0)	0.028
	Both sexes used	-12% (-21, -3)	0.011	-1% (-3, 1)	0.495
D	Sex reported	+8% (-3, 18)	0.165	0% (-2, 2)	0.698
	Both sexes used	+8% (-3, 19)	0.127	+1% (-1, 2)	0.555

Figure 5. Sex reporting and sex of animals used in preclinical studies. (**A**) *Stroke*, (**B**) *Nature Medicine*, (**C**) *Science Translational Medicine* (*Science TM*), and (**D**) control journals. Vertical interrupted lines denote journal interventions. Results shown for sex reporting and use of both sexes. Control journals were analyzed using *Nature Medicine* and *Science TM*'s initiatives as the time series interruption (arrowhead). Δ Level/Slope indicates change in level/slope.

(Intervention to Improve Compliance with the ARRIVE guidelines) study[22] randomized articles reporting in vivo animal research submitted to *PLoS One* to journal-requested completion of the ARRIVE (Animal Research: Reporting of In Vivo Experiments)[29] checklist versus standard practice (no checklist). Contrasting with our results and those of the NPQIP study, the study found no difference in the proportion of articles fully complying with the ARRIVE guidelines. However, the primary outcome in IICARus was reporting compliance with *all* 38 items in the checklist, which authors may have deemed onerous or judged as unlikely to be enforced.

The journal interventions included in the present analysis improved the reporting and use of SDEs to varying degrees. Differences in the nature of the initiatives may account for some of this. For instance, the checklist introduced by *Nature* journals requires authors to describe whether and how specific SDEs were used.[18] In contrast, *Stroke*'s Basic Science Checklist, when first introduced, was a simple and brief questionnaire consisting of dropdown menus with limited options[19] (although it has since been updated to a more comprehensive version[30]). *Science TM* does not have a readily accessible checklist for authors, but instead provides instructions in its Information for Authors section, which its Editor has stated are enforced.[20] Differences in how vigorously journals enforce their requirements and how carefully they screen for author adherence are also be expected to be influential, could fluctuate over time, and may be affected by how attuned reviewers and editors are to them.

The use of key SDEs overall remains low in preclinical research, even in recent years and despite the prominence of the journals examined—a fact that is more clearly brought to light with improved reporting. Based on reported methods, ≈40% to 50% did not use any randomization, >50% did not use any blinding, and up to 75% did not ensure adequate statistical power. Additionally, consistent across journals was a lack of improvement in the inclusion of animals of both sexes after accounting for baseline secular trends. Despite the National Institutes of Health requiring sex inclusion plans in preclinical research funding applications and the importance placed on sex considerations in clinical research, existing journal reporting requirements place little emphasis on this aspect of study design for preclinical experiments.

There are several limitations to our study. The journals examined are widely considered leading translational research journals in terms of impact metrics and reputation. It is possible that their initiatives are more likely to be accepted and adhered to by the research community. The control journals are focused on cardiovascular research. Whether improvements have occurred in noncardiovascular journals that have not implemented interventions analogous to those examined in our study is unknown. However, similar conclusions were reached by the NPQIP Collaborative group using a more heterogeneous control group.[21] Our interrupted time series study design suggests but does not prove causal effects of the interventions examined. However, *Nature Medicine* and *Science TM* are among the few translational research journals that have endorsed the National Institutes of Health guidelines on rigor and reproducibility[11] and communicated to the research community their initiatives with specific implementation dates,[18,20] rendering them ideal case study candidates. In addition, we show a lack of meaningful improvement in the reporting or conduct of preclinical research practices in high-ranking journals that did not implement similar initiatives over the same period despite having similar impact factors to *Science TM*, while confirming previously suggested improvements in *Stroke* after the implementation of analogous initiatives.[12,19] Furthermore, all initiatives addressed randomization, blinding, and sample size estimation, but minimally addressed considering sex as a biological variable, which corresponded to the patterns of improvements seen. Our analysis relied on published reports, therefore the risk of misclassification exists. For instance, the method of randomization used was rarely described, raising the possibility that certain articles that reported randomly assigning animals may have referred to haphazard assignment. Lastly, the study protocol was not registered.

As gatekeepers of what is published, journals' interventions can meaningfully influence the quality of preclinical research and are promising strategies to improve study transparency and design. However, although the studied initiatives were associated with substantial improvements in reporting, improvements in the use of key SDEs were relatively modest, suggesting that additional strategies focused on improving preclinical study design are required to address widespread methodological shortcomings, including sex bias.

Sources of Funding

Dr Ramirez was supported by a Canadian Institutes of Health Research (CIHR) Frederick Banting & Charles Best Canada Graduate Scholarship, a CIHR Banting Postdoctoral Fellowship, the University of Ottawa Heart Institute Foundation, the Ernest and Margaret Ford Research Endowed Fund, and the Cardiac Arrhythmia Network of Canada (CANet) as part of the Networks of Centres of Excellence. R.G. Jung was supported by a CIHR Vanier Canada Graduate Scholarship. Dr Graham was supported by a CIHR Foundation grant (FDN number 143237).

Disclosures

Dr Provencher reports having received grants and personal fees from Actelion Pharmaceuticals, grants from AstraZeneca, and grants from Resverlogix outside the submitted work. Dr Hibbert reports funding as a clinical trial investigator from Abbott and Edwards Lifesciences outside the submitted work. The other authors report no conflicts.

References

1. Collins FS, Tabak LA. Policy: NIH plans to enhance reproducibility. *Nature*. 2014;505:612–613. doi: 10.1038/505612a
2. Landis SC, Amara SG, Asadullah K, Austin CP, Blumenstein R, Bradley EW, et al. A call for transparent reporting to optimize the predictive value of preclinical research. *Nature*. 2012;490:187–191. doi: 10.1038/nature11556
3. Macleod MR, van der Worp HB, Sena ES, Howells DW, Dirnagl U, Donnan GA. Evidence for the efficacy of NXY-059 in experimental focal cerebral ischaemia is confounded by study quality. *Stroke*. 2008;39:2824–2829. doi: 10.1161/STROKEAHA.108.515957
4. Macleod MR, O'Collins T, Howells DW, Donnan GA. Pooling of animal experimental data reveals influence of study design and publication bias. *Stroke*. 2004;35:1203–1208. doi: 10.1161/01.STR.0000125719.25853.20
5. Ioannidis JP, Greenland S, Hlatky MA, Khoury MJ, Macleod MR, Moher D, et al. Increasing value and reducing waste in research design, conduct, and analysis. *Lancet*. 2014;383:166–175. doi: 10.1016/S0140-6736(13)62227-8
6. Freedman LP, Cockburn IM, Simcoe TS. The economics of reproducibility in preclinical research. *PLoS Biol*. 2015;13:e1002165. doi: 10.1371/journal.pbio.1002165
7. van der Worp HB, Howells DW, Sena ES, Porritt MJ, Rewell S, O'Collins V, et al. Can animal models of disease reliably inform human studies? *PLoS Med*. 2010;7:e1000245. doi: 10.1371/journal.pmed.1000245
8. Kilkenny C, Parsons N, Kadyszewski E, Festing MF, Cuthill IC, Fry D, et al. Survey of the quality of experimental design, statistical analysis and reporting of research using animals. *PLoS One*. 2009;4:e7824. doi: 10.1371/journal.pone.0007824
9. Begley CG, Ellis LM. Drug development: raise standards for preclinical cancer research. *Nature*. 2012;483:531–533. doi: 10.1038/483531a
10. Horn J, de Haan RJ, Vermeulen M, Luiten PG, Limburg M. Nimodipine in animal model experiments of focal cerebral ischemia: a systematic review. *Stroke*. 2001;32:2433–2438. doi: 10.1161/hs1001.096009
11. National Institutes of Health. Principles and guidelines for reporting preclinical research. Available at https://www.nih.gov/research-training/rigor-reproducibility/principles-guidelines-reporting-preclinical-research. Accessed July 29, 2019.
12. Ramirez FD, Motazedian P, Jung RG, Di Santo P, MacDonald ZD, Moreland R, et al. Methodological rigor in preclinical cardiovascular studies: targets to enhance reproducibility and promote research translation. *Circ Res*. 2017;120:1916–1926. doi: 10.1161/CIRCRESAHA.117.310628
13. Clayton JA, Collins FS. Policy: NIH to balance sex in cell and animal studies. *Nature*. 2014;509:282–283. doi: 10.1038/509282a
14. Ramirez FD, Motazedian P, Jung RG, Di Santo P, MacDonald Z, Simard T, et al. Sex bias is increasingly prevalent in preclinical cardiovascular research: implications for translational medicine and health equity for women: a systematic assessment of leading cardiovascular journals over a 10-year period. *Circulation*. 2017;135:625–626. doi: 10.1161/CIRCULATIONAHA.116.026668
15. Begley CG, Ioannidis JP. Reproducibility in science: improving the standard for basic and preclinical research. *Circ Res*. 2015;116:116–126. doi: 10.1161/CIRCRESAHA.114.303819
16. Provencher S, Archer SL, Ramirez FD, Hibbert B, Paulin R, Boucherat O, et al. Standards and methodological rigor in pulmonary arterial hypertension preclinical and translational research. *Circ Res*. 2018;122:1021–1032. doi: 10.1161/CIRCRESAHA.117.312579
17. Bolli R. New initiatives to improve the rigor and reproducibility of articles published in Circulation Research. *Circ Res*. 2017;121:472–479. doi: 10.1161/CIRCRESAHA.117.311678
18. Reducing our irreproducibility. *Nature*. 2013;496:398.
19. Minnerup J, Zentsch V, Schmidt A, Fisher M, Schäbitz WR. Methodological quality of experimental stroke studies published in the Stroke journal: time trends and effect of the basic science checklist. *Stroke*. 2016;47:267–272. doi: 10.1161/STROKEAHA.115.011695
20. Kelner KL. Playing our part. *Sci Transl Med*. 2013;5:190ed197
21. The NPQIP Collaborative Group. Did a change in Nature journals' editorial policy for life sciences research improve reporting? *BMJ Open Science*. 2019;3:e000035.
22. Hair K, Macleod MR, Sena ES; IICARus Collaboration. A randomised controlled trial of an Intervention to Improve Compliance with the ARRIVE guidelines (IICARus). *Res Integr Peer Rev*. 2019;4:12. doi: 10.1186/s41073-019-0069-3
23. Baker D, Lidster K, Sottomayor A, Amor S. Two years later: journals are not yet enforcing the ARRIVE guidelines on reporting standards

for pre-clinical animal studies. *PLoS Biol.* 2014;12:e1001756. doi: 10.1371/journal.pbio.1001756
24. Stroke Therapy Academic Industry Roundtable. Recommendations for standards regarding preclinical neuroprotective and restorative drug development. *Stroke*. 1999;30:2752–2758. doi 10.1161/01.str.30.12.2752
25. Unger EF. All is not well in the world of translational research. *J Am Coll Cardiol*. 2007;50:738–740. doi: 10.1016/j.jacc.2007.04.067
26. Kimmelman J, Mogil JS, Dirnagl U. Distinguishing between exploratory and confirmatory preclinical research will improve translation. *PLoS Biol.* 2014;12:e1001863. doi: 10.1371/journal.pbio.1001863
27. Ramirez FD, Hibbert B. Letter by Ramirez and Hibbert regarding article, "Consideration of sex differences in design and reporting of experimental arterial pathology studies: a statement from the Arteriosclerosis, Thrombosis, and Vascular Biology Council." *Arterioscler Thromb Vasc Biol.* 2018;38:e99–e100. doi: 10.1161/ATVBAHA.118.310942
28. Checklists work to improve science. *Nature*. 2018;556:273–274.
29. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol.* 2010;8:e1000412. doi: 10.1371/journal.pbio.1000412
30. Vahidy F, Schäbitz WR, Fisher M, Aronowski J. Reporting standards for preclinical studies of stroke therapy. *Stroke*. 2016;47:2435–2438. doi: 10.1161/STROKEAHA.116.013643

Appendix M

Female authorship in preclinical cardiovascular research: temporal trends and influence on experimental design

Citation information:

Labinaz A, Marbach JA, **Jung RG**, Moreland R, Motazedian P, Di Santo P, Clancy AA, MacDonald Z, Simard T, Hibbert B. Female authorship in preclinical cardiovascular research: temporal trends and influence on experimental design. (**2019**). *JACC: Basic to Translational Science.*

JACC: BASIC TO TRANSLATIONAL SCIENCE VOL. 4, NO. 4, 2019
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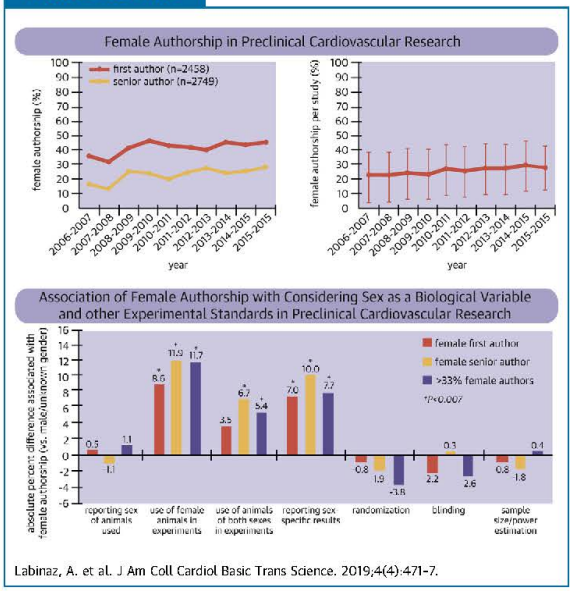
PRECLINICAL RESEARCH

Female Authorship in Preclinical Cardiovascular Research

Temporal Trends and Influence on Experimental Design

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VISUAL ABSTRACT



Labinaz, A. et al. J Am Coll Cardiol Basic Trans Science. 2019;4(4):471-7.

HIGHLIGHTS

- In this analysis of 3,396 preclinical studies published in 5 leading cardiovascular journals over a 10-year period, women accounted for 24 ± 17% of authors per manuscript.
- Female authorship is increasing in preclinical cardiovascular science, but the proportions of articles with first and senior authors of different sex have remained unchanged, which suggests that segregation by sex in mentorship relationships exists and persists.
- In preclinical studies that reported the sex of the animals used, female authorship was positively associated with studying female animals, using animals of both sexes, and reporting sex-specific results, which are findings that persisted in adjusted and sensitivity analyses.
- Author sex was not associated with other measures of methodological rigor or with 60-month citation counts.

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ISSN 2452-302X https://doi.org/10.1016/j.jacbts.2019.04.004

JACC: BASIC TO TRANSLATIONAL SCIENCE VOL. 4, NO. 4, 2019
AUGUST 2019:471–7

ABBREVIATIONS AND ACRONYMS

CI = confidence interval

NIH = National Institutes of Health

OR = odds ratio

SUMMARY

In this analysis of 3,396 preclinical cardiovascular studies, women were first, senior, and both first and senior authors in 41.3%, 20.7%, and 11.0% of the studies, respectively. Female authorship increased over a 10-year period. However, the proportion of studies with first and senior authors of differing sex was low and stable, suggesting that segregation by sex in mentorship relationships exists and persists. Female authors were more likely to consider sex as a biological variable, but author sex was not associated with other measures of experimental rigor or research impact, indicating that women's underrepresentation was not due to differences in research capacity or impact. (J Am Coll Cardiol Basic Trans Science 2019;4:471–7) © 2019 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

The now outdated axiom that cardiovascular disease is a disease of men began to be meaningfully challenged in the 1980s, with considerable effort focused on adequately representing women in clinical trials. Central to many of these initiatives was (and continues to be) the recognition that fundamental yet poorly understood differences exist between men and women, and that these differences could engender health disparities if ignored. However, this "revolution" has not permeated preclinical stages of research, which serve to inform clinical trials. The preferential use of male animals and a lack of sex-disaggregated reporting is increasing in cardiovascular science (1), despite the emphasis of the National Institutes of Health (NIH) on considering sex in preclinical research as important for advancing the health of women (2) and despite its feasibility in most research settings (3). This bias has the potential to undermine advances made in clinical trial design by skewing our understanding of disease processes and the effectiveness of potential therapies (4).

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It has been argued that the participation of women in research enhances knowledge outcomes and scientific progress (5), in part via enhanced exploration of sex differences (6). If true, sex gaps in medicine and research—including the underrepresentation of women in fields such as cardiology (7) and the relative lack of adequate mentorship for women (8)—would not only have important societal implications but also meaningful scientific ramifications. Recognizing this possibility, the NIH has emphasized their commitment to diversity in the biomedical research workforce (including better representation of women) and identified assessing the impact of this diversity on the quality and outputs of research as 1 of 4 major diversity challenges facing the biomedical "ecosystem" (9).

We therefore examined a large body of leading preclinical cardiovascular research to explore potential differences in experimental rigor between male and female researchers, focusing on the inclusion of female animals in experiments and on analyses of sex differences. Temporal trends in female authorship and patterns in mentorship relationships by sex were examined as secondary analyses. We hypothesized that female authorship and mentorship relationships between men and women had both increased, but that these would not be associated with increased consideration of sex as a biological variable or with other measures of experimental rigor.

METHODS

As described (10), all preclinical studies published between July 2006 and June 2016 in *Circulation*; *Circulation Research*; *Hypertension*; *Stroke*; and *Arteriosclerosis, Thrombosis, and Vascular Biology* were reviewed. Studies were included if they reported original data from in vivo experiments using nonhuman mammals and proposed therapeutic applications or implications to specific human disorders. Pre-specified variables collected included the disease studied, the animal model(s) used and their sex, and whether any study result was reported by sex. Because of the possibility that differences in female animal inclusion or sex-specific analyses might be attributable to broader differences in experimental design, we also analyzed whether animals were randomized, whether blinding was used (concealed allocation or

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Manuscript received March 13, 2019; revised manuscript received April 22, 2019, accepted April 27, 2019.

blinded outcome assessment), and whether sample size and/or power estimations were performed.

Study author names were extracted from Scopus. Author sex was determined using the online database genderize.io, which included >216,000 first names across 79 countries and 89 languages when queried. The database determines the sex of a name with an associated certainty factor. First authors are generally considered to have executed most of the published work, whereas senior authors are usually the study supervisors or principal investigators, and are generally considered to have contributed most to study planning and design (11,12); therefore, these were selected as our primary analyses. Female authorship as a proportion of all authors per manuscript was examined as a secondary analysis (per 10% increase and dichotomized as ≥33% of authors). Scopus was also queried to determine citation counts at 60 months for articles published between July 2006 and June 2011, as described (10). Post hoc analyses of mentorship relationships by sex were performed, with senior authors designated as mentors and first authors as mentees. A certainty factor of ≥70% was used to assign author sex for all analyses, otherwise sex was considered unknown. Articles with authors of unknown sex were excluded in primary analyses but were included in secondary and post hoc analyses to minimize missing data. Sensitivity analyses using a certainty factor of ≥90% for author sex and of sex-specific reporting restricted to studies in which animals of both sexes were used were performed.

Categorical variables are reported as number (percentage) and were compared using chi-square tests. Continuous variables are reported as mean ± SD or median with interquartile range (25th to 75th percentiles) (IQR) and were compared using Student's *t*-tests or Mann-Whitney U tests, respectively. Temporal trends were assessed using Cochran-Armitage tests or Spearman's rank-order correlation (ρ) using 12-month intervals. The associations of author sex with factoring sex of the animals studied in the reporting, design, and analysis of experiments and with the implementation of other study design elements were examined using chi-square tests (reported as absolute percent differences in study characteristics) and via simple and multivariable logistic regression (reported as odds ratios [ORs] with 95% confidence intervals [CIs]). Citation count comparisons were performed via stratification and multivariable linear regression. All analyses were performed using SAS version 9.4 (SAS Institute, Inc., Cary, North Carolina) using a 2-tailed α level of 0.05 (corrected using the Bonferroni method to account for multiple comparisons when specified).

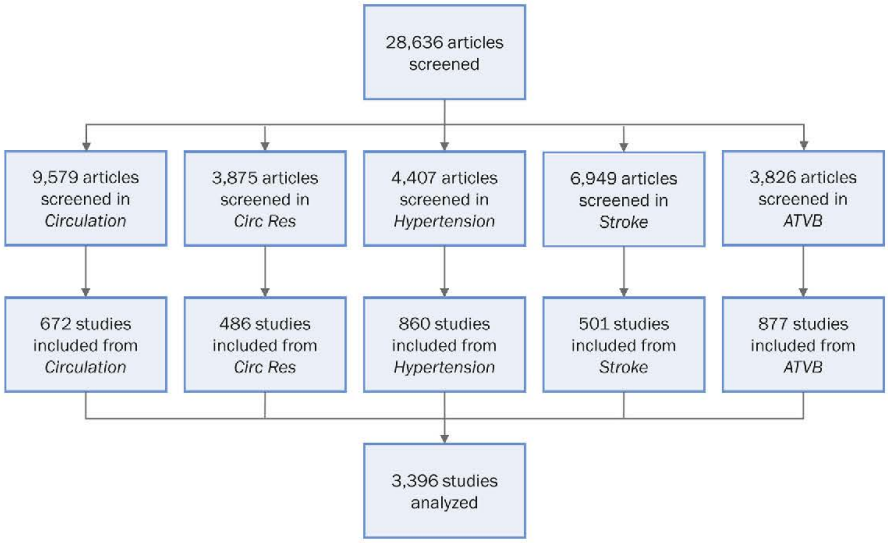
RESULTS

Of 28,636 articles screened, 3,396 met the study inclusion criteria (**Figure 1**). Women accounted for 24 ± 17% of authors per manuscript, with 542 articles (16.0%) not including any female authors and none being authored solely by women. After excluding articles with authors of unknown sex, women were identified as first authors of 1,016 of 2,458 articles, senior authors of 569 of 2,749 articles, and both first and senior authors of 235 of 2,135 preclinical studies (41.3%, 20.7%, and 11.0%, respectively). The distribution of observed mentorship relationships differed from the expected distribution, with disproportionately high numbers of same-sex mentorships identified ($p < 0.001$), including a relative 50% greater than expected frequency of male to male mentorships. No difference was observed in the number of coauthors of articles with female versus male first or senior authors (9.6 vs. 9.6; $p = 0.864$; and 9.2 vs. 9.5; $p = 0.075$; respectively). Five-year citation counts were comparable between female and male authors (21 [interquartile range (IQR): 13 to 31] vs. 20 [IQR: 12 to 33]; $p = 0.509$ for first authors; 18 [IQR: 12 to 30] vs. 20 [IQR: 13 to 31]; $p = 0.170$ for senior authors). Author sex was similarly not predictive of citation counts in multivariable models that adjusted for cardiovascular disease studied, publication date, and journal.

Temporal analyses over 10 years indicate that the proportions of female first and senior authors each increased, with women accounting for between 32.3% and 46.3% of first authors and between 11.8% and 26.1% of senior authors (**Figure 2A**). There was a corresponding temporal increase in the proportion of manuscripts with women as both first and senior authors (full range: 5.5% to 14.9%; $p_{trend} = 0.001$). The proportions of articles with first and senior authors of differing sex did not change (full range: 28.5% to 34.3%; $p_{trend} = 0.663$ for female first and male senior authorship; full range: 5.5% to 14.9%; $p_{trend} = 0.184$ for male first and female senior authorship). Per article, the mean proportion of female authors slightly increased (full range: 21.4% to 26.8%; $p < 0.001$) (**Figure 2B**).

Female and male authors were equally likely to report the sex of animals used in preclinical experiments (815 of 1,016 [80.2%] vs. 1,150 of 1,442 [79.8%]; $p = 0.776$ for first authors; 455 of 569 [80.0%] vs. 1,767 of 2,180 [81.1%]; $p = 0.556$ for senior authors). However, when the sex of the animals was reported, women were more likely to have used female animals in their experiments (292 of 815 [35.8%] vs. 313 of 1,150 [27.2%], $p < 0.001$ for first authors; 181 of 455 [39.8%] vs. 492 of 1,767 [27.8%]; $p < 0.001$ for senior

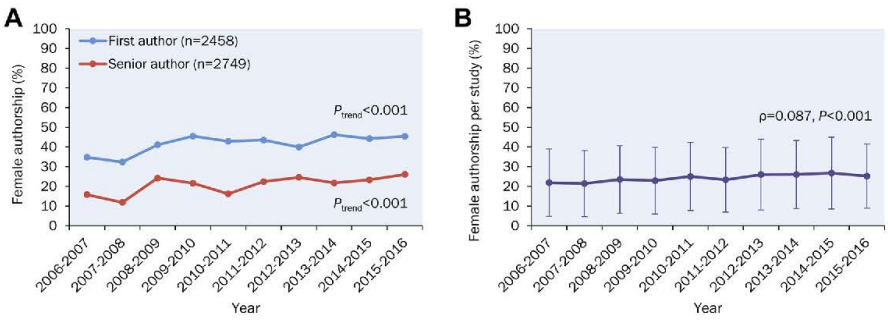
FIGURE 1 Literature Search



Literature search. *ATVB* = *Arteriosclerosis, Thrombosis and Vascular Biology*; *Circ Res* = *Circulation Research*. Modified from Ramirez et al. (10).

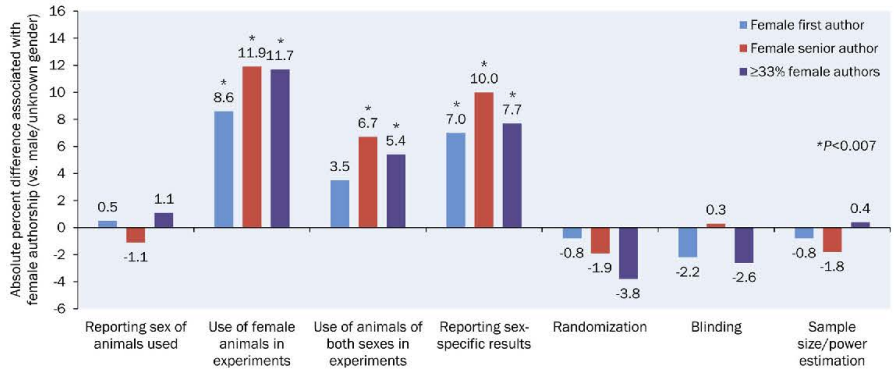
authors), to have used animals of both sexes (156 of 815 [19.1%] vs. 179 of 1,150 [15.6%]; p = 0.038 for first authors; 98 of 455 [21.5%] vs. 262 of 1,767 [14.8%]; p < 0.001 for senior authors), and to have reported sex-specific results (326 of 815 [40.0%] vs. 380 of 1,150 [33.0%]; p = 0.002 for first authors; 199 of 455 [43.7%] vs. 597 of 1,767 [33.8%]; p < 0.001 for senior authors). Similar findings were observed when female authorship was examined as a proportion of all authors per manuscript. In contrast, female authorship was not associated with randomization, blinding, or sample size estimation after correcting for multiple

FIGURE 2 Female Authorship in Preclinical Cardiovascular Research



(A) Proportion of women as first and senior authors. **(B)** Mean proportion of women as authors per study. Error bars depict SD.

FIGURE 3 Association of Female Authorship With Considering Sex as a Biological Variable and Other Experimental Standards in Preclinical Cardiovascular Research



Absolute percent differences shown for first, senior, and ≥33% female authorship. *Corrected using the Bonferroni method to account for multiple comparisons ($\alpha = 0.05$ divided by 7).

comparisons (Figure 3). The preceding differences persisted when female authorship was examined on an interval scale (per 10% increase) and after adjusting for pre-specified potential confounders (Table 1). All findings were also comparable in sensitivity analyses using a minimum certainty factor of 90% for author sex. When restricted to studies that included both male and female animals (N = 335 to 421), female senior authorship (but not first or cumulative authorship) remained associated with reporting sex-specific results after adjusting for pre-specified potential confounders (OR: 2.2; 95% CI: 1.1 to 4.4; p = 0.036).

DISCUSSION

Sex differences in innate scientific ability have long ago been refuted (13); however, career trajectories and personal and professional experiences often differ between male and female academics (8,13), which may influence researchers' priorities and practices. Although there are clear societal benefits to promoting sex inclusivity in research, its impact on the quality and impact of research remains unexplored (9). Our analysis of preclinical cardiovascular research demonstrates that: 1) approximately 41% of first authors and 21% of senior authors over a recent 10-year period were women; 2) although female authorship is increasing, segregation by sex in mentorship relationships exists and persists; and 3) when women influence experimental design and reporting or are a larger proportion of the research team, studies are more likely to include female animals and to explore sex-based differences, but author sex is not associated with other measures of methodological rigor or research impact.

Preclinical research using animal models often precedes and informs clinical trials. However, important and remediable methodological shortcomings remain prevalent in preclinical cardiovascular research (10), including a tendency to exclude females in animal experiments and to ignore the potential influence of sex on study outcomes (1). Women's perspectives have been suggested to uniquely promote scientific progress in general and advances in women's health in particular, in part due to their greater tendency to explore the effects of gender and sex (5,6). Our data suggest that women are indeed more attuned to considering sex as a biological variable in preclinical experiments, but that male and female researchers are more alike than they are different when broader measures of scientific rigor and research impact are considered.

A corollary to the previously described findings is that the persistent underrepresentation of women in preclinical cardiovascular research is highly unlikely to be attributable to differences in research capacity or potential impact. Rather, systemic factors are probably influential. For instance, although modern scientific endeavors are increasingly reliant on research teams and networks—settings in which a diversity of viewpoints and experiences are sought

TABLE 1 Association Between Female Authorship and Experimental Design Characteristics in Preclinical Cardiovascular Studies

	N*	Crude OR (95% CI)	Adjusted OR (95% CI)†	p Value†
Female first author				
Reporting sex of animals	2,458	1.03 (0.84–1.26)	0.96 (0.77–1.19)	0.698
Inclusion of female animals‡	1,965	1.49 (1.23–1.81)	1.60 (1.30–1.96)	<0.001
Inclusion of animals of both sexes‡	1,965	1.28 (1.01–1.63)	1.29 (1.01–1.66)	0.046
Sex-specific reporting of results‡	1,965	1.35 (1.12–1.63)	1.33 (1.10–1.62)	0.004
Randomization	2,458	0.95 (0.78–1.16)	1.03 (0.83–1.28)	0.776
Blinding	2,458	0.91 (0.76–1.08)	0.95 (0.79–1.15)	0.608
Sample size estimation	2,458	0.70 (0.41–1.20)	NR	
Female senior author				
Reporting sex of animals	2,749	0.93 (0.74–1.18)	0.83 (0.65–1.07)	0.153
Inclusion of female animals‡	2,222	1.71 (1.38–2.12)	1.81 (1.43–2.28)	<0.001
Inclusion of animals of both sexes‡	2,222	1.58 (1.22–2.04)	1.58 (1.20–2.08)	0.001
Sex-specific reporting of results‡	2,222	1.52 (1.24–1.88)	1.44 (1.16–1.79)	0.001
Randomization	2,749	0.90 (0.71–1.12)	0.88 (0.68–1.13)	0.308
Blinding	2,749	1.02 (0.84–1.23)	1.14 (0.92–1.42)	0.219
Sample size estimation	2,749	0.32 (0.13–0.80)	NR	
Female authorship (per 10% increase)				
Reporting sex of animals	3,396	1.04 (0.98–1.09)	1.02 (0.97–1.09)	0.435
Inclusion of female animals‡	2,718	1.20 (1.14–1.26)	1.20 (1.13–1.26)	<0.001
Inclusion of animals of both sexes‡	2,718	1.13 (1.07–1.21)	1.12 (1.05–1.20)	<0.001
Sex-specific reporting of results‡	2,718	1.13 (1.08–1.19)	1.11 (1.05–1.17)	<0.001
Randomization	3,396	0.95 (0.90–1.00)	1.00 (0.94–1.06)	0.949
Blinding	3,396	0.99 (0.94–1.03)	1.04 (0.99–1.09)	0.173
Sample size estimation	3,396	1.08 (0.94–1.24)	NR	

*Refers to the total number of studies included in analyses. †Adjusted for disease studied, animal model(s) used, journal of publication, date of publication, and number of co-authors. ‡Analysis restricted to studies in which the sex of animals used was reported. Bonferroni corrected α level of 0.007 used to define statistical significance (α = 0.05 divided by 7).

CI = confidence interval; NR = not reported due to small number of events per predictor variable; OR = odds ratio.

and valued (6)—our analysis highlights a persistent predominance of same-sex mentorships. Because of the relative paucity of available female mentors, this practice has the potential to perpetuate women's underrepresentation in the field and to hinder efforts to improve the status quo (7).

STUDY LIMITATIONS. The journals examined were selected based on their prominence in cardiovascular research, their collective focus on a wide range of cardiovascular disorders, and their endorsement of NIH guidelines on rigor and reproducibility (10). However, they might not be representative of all preclinical cardiovascular journals. Author sex was determined using an arbitrary certainty factor, which might have resulted in misclassification in a minority of cases. However, our results were comparable in sensitivity analyses using a more stringent criterion. Our analysis did not capture instances of multiple first or corresponding authors (equally contributing authors) and used author position as a proxy for mentor–mentee relationships. Presumed author sex might not reflect author gender, which might be a relevant distinction in our analysis. Our analysis also focused on experimental and reporting standards proposed by the NIH, which were not exhaustive.

CONCLUSIONS

Women's involvement in preclinical cardiovascular research is positively associated with considering sex as a biological variable, which is a practice that is expected to inform and promote advances in women's health. Researcher sex is not associated with other measures of experimental rigor or research impact. Limited mentorship opportunities may be contributing to women's underrepresentation in this field.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: In this analysis of 3,396 preclinical studies in 5 leading cardiovascular journals, female authorship was positively associated with considering sex as a biological variable, but not with other measures of methodological rigor or 60-month citation counts. Over a 10-year period, the proportion of studies with first and senior authors of differing sex was low and stable, suggesting that segregation by sex in mentorship relationships exists and persists. These data indicate that women's underrepresentation in preclinical cardiovascular research is not due to differences in research capacity or impact. Limited mentorship opportunities for women in preclinical cardiovascular research may be an important contributor.

TRANSLATIONAL OUTLOOK: Further study to identify and understand barriers to women's involvement in preclinical cardiovascular research is warranted. Enhancing mentorship opportunities for women should be explored as a potential strategy to improve the status quo.

REFERENCES

1. Ramirez FD, Motazedian P, Jung RG, et al. Sex bias is increasingly prevalent in preclinical cardiovascular research: implications for translational medicine and health equity for women: a systematic assessment of leading cardiovascular journals over a 10-year period. Circulation 2017;135:625–6.

2. Clayton JA, Collins FS. Policy: NIH to balance sex in cell and animal studies. Nature 2014;509:282–3.

3. Beery AK. Inclusion of females does not increase variability in rodent research studies. Curr Opin Behav Sci 2018;23:143–9.

4. Klein SL, Schiebinger L, Stefanick ML, et al. Opinion: sex inclusion in basic research drives discovery. Proc Natl Acad Sci U S A 2015;112:5257–8.

5. Rotenstein LS, Jena AB. Lost Taussigs - the consequences of gender discrimination in medicine. N Engl J Med 2018;378:2255–7.

6. Nielsen MW, Alegria S, Borjeson L, et al. Opinion: gender diversity leads to better science. Proc Natl Acad Sci U S A 2017;114:1740–2.

7. Asghar M, Usman MS, Aibani R, et al. Sex Differences in authorship of academic cardiology literature over the last 2 decades. J Am Coll Cardiol 2018;72:681–5.

8. Soklaridis S, Zahn C, Kuper A, Gillis D, Taylor VH, Whitehead C. Men's fear of mentoring in the #MeToo era - what's at stake for academic medicine? N Engl J Med 2018.

9. Valantine HA, Collins FS. National Institutes of Health addresses the science of diversity. Proc Natl Acad Sci U S A 2015;112:12240–2.

10. Ramirez FD, Motazedian P, Jung RG, et al. Methodological rigor in preclinical cardiovascular studies: targets to enhance reproducibility and promote research translation. Circ Res 2017;120:1916–26.

11. Wren JD, Kozak KZ, Johnson KR, Deakyne SJ, Schilling LM, Dellavalle RP. The write position. A survey of perceived contributions to papers based on byline position and number of authors. EMBO Rep 2007;8:988–91.

12. Lariviere V, Desrochers N, Macaluso B, Mongeon P, Paul-Hus A, Sugimoto CR. Contributorship and division of labor in knowledge production. Soc Stud Sci 2016;46:417–35.

13. Ceci SJ, Ginther DK, Kahn S, Williams WM. Women in academic science: a changing landscape. Psychol Sci Public Interest 2014;15:75–141.

KEY WORDS cardiology, mentorship, translational research, women

Appendix N

A shifting paradigm – the role of focused cardiac ultrasound in bedside patient assessment

Citation information:

Marbach JA, Almufleh A, Di Santo P, Simard T, **Jung RG**, Diemer G, West FM, Millington SJ, Mathew R, Le May M, Hibbert B. A shifting paradigm – the role of focused cardiac ultrasound in bedside patient assessment. (**2020**). *Chest*.

Addendum:

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Appendix O

Evaluation of an in vitro coronary stent thrombosis model for preclinical assessment

Citation information:

Jung RG*, Perry-Nguyen D***,** Labinaz A, Duchez AC, Dewidar O, Simard T, Karunakaran D, Majeed K, Sarathy K, Li R, Ramirez FD, Di Santo P, Rochman R, So D, Foin N, Hibbert B. Evaluation of an *in vitro* coronary stent thrombosis model for preclinical assessment. (**2019**). *Platelets.*

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Evaluation of an in vitro coronary stent thrombosis model for preclinical assessment

Author: Dylan Perry-Nguyen, , Richard G. Jung, et al
Publication: PLATELETS
Publisher: Taylor & Francis
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http://www.tandfonline.com/iplt
ISSN: 0953-7104 (print), 1369-1635 (electronic)

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Platelets, Early Online: 1–7
© 2019 Taylor & Francis Group, LLC. DOI: https://doi.org/10.1080/09537104.2019.1595564

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METHODS PAPER

Check for updates

Evaluation of an *in vitro* coronary stent thrombosis model for preclinical assessment

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Abstract

Stent thrombosis remains an infrequent but significant complication following percutaneous coronary intervention. Preclinical models to rapidly screen and validate therapeutic compounds for efficacy are lacking. Herein, we describe a reproducible, high throughput and cost-effective method to evaluate candidate therapeutics and devices for either treatment or propensity to develop stent thrombosis in an *in vitro* bench-top model. Increasing degree of stent malapposition (0.00 mm, 0.10 mm, 0.25 mm and 0.50 mm) was associated with increasing thrombosis and luminal area occlusion (4.1 ± 0.5%, 6.3 ± 0.5%, 19.7 ± 4.5%, and 92.6 ± 7.4%, $p < 0.0001$, respectively). Differences in stent design in the form of bare-metal, drug-eluting, and bioresorbable vascular scaffolds demonstrated differences in stent thrombus burden (14.7 ± 3.8% vs. 20.5 ± 3.1% vs. 86.8 ± 5.3%, $p < 0.01$, respectively). Finally, thrombus burden was significantly reduced when healthy blood samples were incubated with Heparin, ASA/Ticagrelor (DAPT), and Heparin+DAPT compared to control (DMSO) at 4.1 ± 0.6%, 6.9 ± 1.7%, 4.5 ± 1.2%, and 12.1 ± 1.8%, respectively ($p < 0.01$). The reported model produces high throughput reproducible thrombosis results across a spectrum of antithrombotic agents, stent design, and degrees of apposition. Importantly, performance recapitulates clinical observations of antiplatelet/antithrombotic regimens as well as device and deployment characteristics. Accordingly, this model may serve as a screening tool for candidate therapies in preclinical evaluation.

Keywords

Antiplatelet agents, methods, optical coherence tomography, percutaneous coronary intervention, stent thrombosis

History

Received 9 November 2018
Revised 9 March 2019
Accepted 10 March 2019
Published online 3 April 2019

Introduction

Since the first angioplasty in 1977, percutaneous management of coronary artery disease has greatly evolved [1]. A combination of percutaneous coronary intervention (PCI) with optimal medical therapy has become the standard of care to manage patients presenting with acute coronary syndromes (ACS) and obstructive coronary artery disease (CAD). Despite the temporal improvement in PCI technology, stent thrombosis (ST) and in-stent restenosis [2] continue to complicate revascularization procedures resulting in target lesion failure. Despite the low incidence of stent thrombosis (<1%) in the first-year using second-generation drug-eluting stents, patients with ST typically present with ST-elevation myocardial infarction with mortality rates as high as 20–40% [3,4]. To reduce rates of acute vessel failure due to ST, current medical therapy includes dual antiplatelet therapy (DAPT) including a combination of aspirin and a $P2Y_{12}$ inhibitor for 6 to 12 months [5,6] post-intervention.

While rare, stent thrombosis is a life-threatening complication due to the formation of a thrombus within the stent lumen leading to occlusion and myocardial infarction [7]. Risk factors for ST include patient-related factors, such as diabetes and drug metabolism, and procedural or technical characteristics relating to the site of lesion or stent. From a pathophysiological perspective, ST is linked to Virchow's triad of thrombogenesis and is specifically believed to occur from a combination of improper re-endothelialization of the stented wall leading to exposed stent struts and a pro-thrombotic hemodynamic milieu, particularly relating to platelet activation, adhesion, and subsequent clot formation [8]. Moreover, small stent diameter and malapposition of stents have been associated with ST [9,10]. These factors may result in significant disruptions to cause turbulent flow patterns and wildly variable patterns of recirculation and stagnation along the lumen which serve as a nidus for local thrombosis formation between the wall and strut [11,12]. Models to study ST are lacking, due to a lack of frequency in endpoints in

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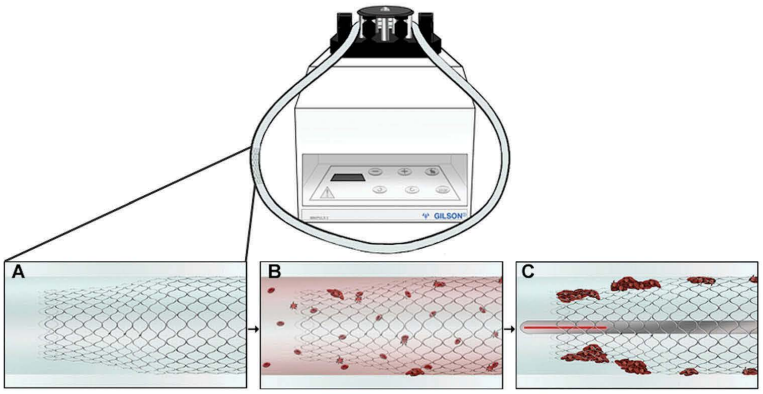


Figure 1. **Diagram of the bench top stent thrombosis model**. Stents were deployed at the manufacturer's recommendation of pressure to desired stent diameter within the silicone tube and set up in a circular loop within the MINIPULS3 peristaltic pump. (**A**) Typical image of stent deployment which was half malapposed and half well apposed along length of the stent. (**B**) As blood perfused through the stent at 50 mL/min for 15 min, platelet activation and thrombus formation could be observed on the stent struts. (**C**) Upon the completion of perfusion, tubes were purged with Omnipaque contrast solution and OCT images of the stent were obtained at a rate of 10 mm/sec over 50 mm lengths.

animal models and poorly validated *in vitro* models to screen for novel therapeutic targets.

Optical coherence tomography (OCT) is an intravascular imaging technique currently used to provide high-resolution anatomical information relating to the coronary artery lesion, the stent or scaffold deployed, and/or to assess for intracoronary thrombus. OCT offers superior resolution to intravascular ultrasound, with anatomical discrimination of structures in the 10-20 μm range [13]. Accordingly, this technology affords high-resolution images and precise quantification of tissue, devices, and thrombus following stent deployment [14].

Herein, we report the evaluation of a standardized *in vitro* ST model using OCT to quantify in-stent thrombus formation across a spectrum of pathology. The model permits permutations in the device, deployment characteristics, and therapeutic antagonism using human whole blood samples. Moreover, by using a standardized assay, patient characteristics such as drug therapy or comorbidities can be evaluated as potential risk factors.

Methods/Protocol

Model Parts and Inhibitors

Antithrombotic agents aspirin, $P2Y_{12}$ inhibitor (Ticagrelor), and Heparin were obtained from Sigma. Corn trypsin inhibitor (CTI) was obtained from Haematologic Technologies (Essex Junction, Vermont, USA). MINIPULS3 peristaltic pump was obtained from Mandel Scientific Company (Guelph, ON, Canada). Tubing and luer locks were obtained from QOSINA Corp. (Ronkonkoma, NY, USA). Endeavor-RX drug-eluting stents and Integrity bare-metal stents were obtained from Medtronic (Minneapolis, MN, USA) while Xience Xpedition drug-eluting stents and Absorb GT1 bioresorbable vascular scaffolds were obtained from Abbott (Chicago, IL, USA). Stents were deployed at manufacturers' recommended pressures for desired stent diameters. Finally, the optical coherence tomography images were obtained using the ILUMIEN OPTIS PCI Optimization system from St. Jude Medical (Saint Paul, MN, USA).

The current model uses 50A Durometer silicone tubing of 3.2 mm ID/6.4mm OD (1/8" ID/1/4"OD) from QOSINA Corp. (Part no. T2013). To form the loops, two lengths of 27 cm and 45 cm (10.6" and 17.7", respectively) were joined using a 3-way stopcock with a thru hole diameter of 2.7 mm (Part no. 88218). Male and female luer lock connectors were attached to the ends of tubing to facilitate proper connection (Part no. 11549 and 11548).

Within the 27-cm portion of tubing, stents were expanded to diameters ranging from 2.5 to 3.0 mm based on manufacturer recommendations. The distal half of the stent was then over-expanded at high pressures to ensure proper apposition to the tubes. The proximal segments were left malapposed as targeted. The diameter of deployed stent segments was visually confirmed and measured via OCT before blood samples were perfused through the model.

Blood Collection and Preparation

Volunteer venous blood was obtained using 21G needles, and patient arterial blood was taken from patients' arterial access sites upon the completion of coronary angiography. Blood was obtained using Vacutainer tubes containing 1% sodium citrate (Becton Dickinson, Franklin Lakes, NJ, USA). Samples were incubated using an automatic nutator for 15 min before 6.5 mL of the sample was injected into the perfusion model. The tubing loop was closed manually, and any excess air was removed through the luer lock using a syringe that contained the remaining sample blood. At room temperature 21°C, the sample was run at a flow rate of 51.0 $\pm$ 1.6 mL/min for 15 min unless otherwise indicated. To examine the effect of antithrombotic agents, samples were incubated with indicated agents for 15 min prior to perfusion. A representative diagram of the model is shown in Figure 1.

Examination of Thrombus Burden

Upon completion of perfusion, Omnipaque contrast (GE Healthcare, Chicago, IL, USA) was circulated through the tubing to expel remaining blood and OCT images of the stented area were obtained at a rate of 10 mm/s over 50 mm. OCT images were subsequently analyzed using the OPTIS workstation to measure the tube and stent diameter along with total thrombus area every 10 frames in a blinded fashion. Areas of malapposition were analyzed every 2 frames due to significantly increased thrombus burden in these areas. The frames flanking areas of notable thrombus build-up were further analyzed to ensure capture of a "maximal occlusion" point. Stent struts were defined as being malapposed if the axial distance between the strut's surface and the luminal surface was greater than the strut thickness in addition to the

axial resolution of OCT [15]. The transitional region from well apposed to malapposed stent segments was defined as transition from 0° to 360° arc-angle of malapposition upon OCT analysis.

Statistics

All continuous variables were reported as mean (±standard error of the mean). Continuous variables were first examined for normality using the Q-Q plot, and subsequently, student t-test, ANOVA, or Tukey test was performed as appropriate. $P < 0.05$ was considered significant. Figures and statistical tests were conducted using GraphPad Prism v7 (GraphPad Software, La Jolla, CA, USA) and SAS v9.4 (SAS Institute, Inc).

Ethics Statement

This study was reviewed and approved by the Ottawa Health Science Network Research Ethics Board (OHSN-REB) and written informed consent was obtained from all volunteers and patients involved. The present study conforms with the 1975 Declaration of Helsinki concerning the experimental use of human blood.

Results

Optimization of Perfusion Parameters

In order to determine the optimal content of sodium citrate, blood from volunteers was collected in 1%, 2%, and 3% sodium citrate tubes and incubated for 15 min prior to analysis. Thrombus burden was significantly larger with blood collected in 1% as opposed to 2% and 3% citrate tubes (20.5 ± 3.1%; n = 16, 5.6 ± 0.5%; n = 6, and 8.1 ± 2.0%; n = 8, for 1%, 2% and 3%, respectively, $p < 0.01$, Figure 2A). Blood collected in 0% sodium citrate tubes thrombosed prior to running the assay and were excluded from the analysis. Furthermore, activated clotting times did not differ between 0%, 1%, and 3% sodium citrate tubes (**Supplemental Figure 1**). Secondarily, perfusion time was optimized targeting 20–80% clot formation. Samples with 1% citrate were run for 10, 15, and 20 min prior to analysis of thrombus burden by OCT. An increase in thrombus burden was observed as perfusion duration increased from 10 to 20 minutes (7.4 ± 1.8%; n = 7, 20.5 ± 3.1%; n = 16, and 23.7 ± 3.2%; n = 7, for 10, 15, and 20 min, respectively, $p = 0.01$, Figure 2B). Thrombus burden stabilized at 20 min with no significant progression from 15 min.

Impact of Stent Apposition and Design on Thrombus Burden

To determine the effect of stent malapposition on thrombus formation, stents were deployed with malapposition in the proximal portion and apposition in the distal portion with respect to the direction of perfusion (n = 15, Figure 1A). When blood was perfused for 15 min through this configuration, thrombus burden was greater in the malapposed segment compared to the well-apposed stent segment (21.3 ± 3.2% vs. 7.0 ± 0.9%, $p < 0.01$, Figure 3A). Samples perfused in the opposite direction showed no difference in thrombus burden (**Supplemental Figure 2**). Next, to evaluate the impact of complete

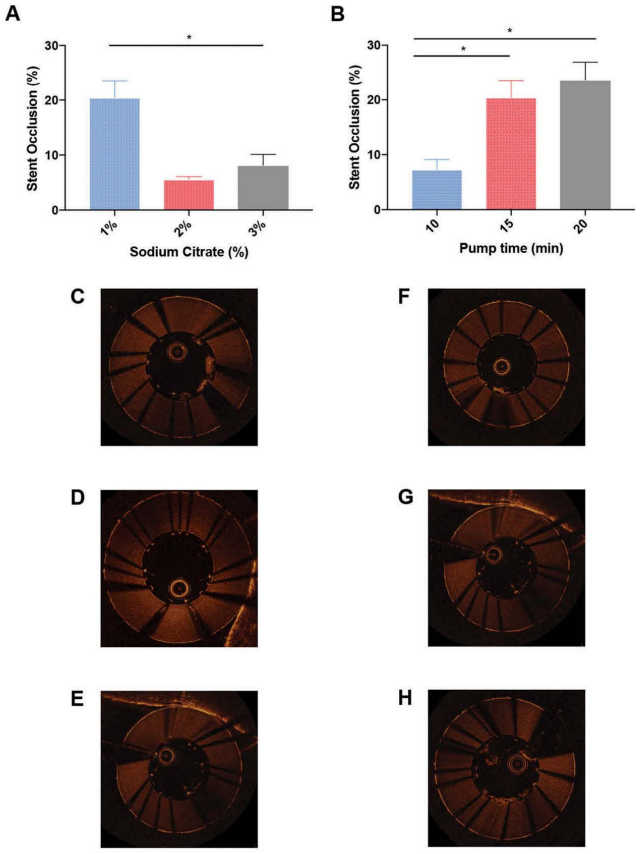


Figure 2. **Optimization of sodium citrate content and perfusion duration**. (**A**) Blood from volunteers was collected in 0%, 1%, 2% and 3% sodium citrate tubes and was incubated for 15 min prior to perfusion through the bench-top stent thrombosis model. Thrombus burden was higher in 1% sodium citrate tubes (20.5 ± 3.1%; n = 16, 5.6 ± 0.5%; n = 6, and 8.1 ± 2.0%; n = 8, $p < 0.01$). Blood obtained in 0% sodium citrate tubes clotted prior to analysis and were excluded for the remainder of the study. (**B**) Blood in 1% citrate tubes were perfused for 10, 15, and 20 min prior to analysis of thrombus burden by OCT. An increase in thrombus burden was observed as the perfusion duration increased from 10 to 20 minutes (7.4 ± 1.8%, 20.5 ± 3.1%, and 23.7 ± 3.2% for 10, 15, and 20 min, respectively, $p < 0.01$). (**C**) Representative OCT image of 1% sodium citrate. (**D**) Representative OCT image of 2% sodium citrate. (**E**) Representative OCT image of 3% sodium citrate. (**F**) Representative OCT image of 10 min of perfusion. (**G**) Representative OCT image of 15 min of perfusion. (**H**) Representative OCT image of 20 min of perfusion. Same data was used for 1% sodium citrate which was perfused for 15 min (Figure 2A – 1% and 2B – 15 min). Data is expressed as mean±standard error of the mean and was analyzed by ANOVA and Tukey Test. $p \leq 0.05$ is considered statistically significant. *P < 0.05; **P < 0.01.

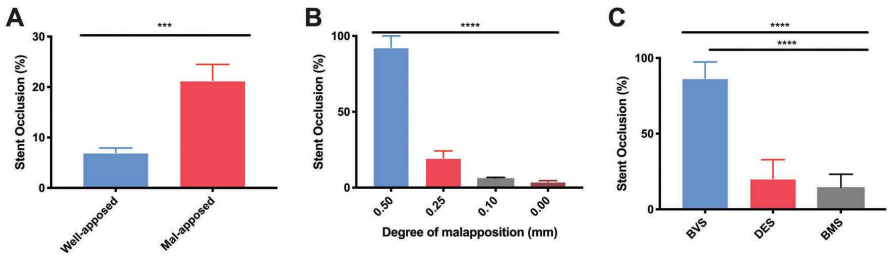


Figure 3. **Effect of malapposition and stent design on thrombus burden.** (**A**) Blood from volunteers collected in 1% sodium citrate tubes was perfused for 15 min through a stent which was half malapposed (0.25 mm) and half well-apposed (0.00 mm) from the lumen walls. Thrombus burden was higher in the malapposed segment compared to the well-apposed segment (21.3 ± 3.2% vs. 7.0 ± 0.9%, $p < 0.01$). (**B**) Blood from volunteers was perfused for 15 min through stents with varying degrees of malapposition relative to the lumen wall. Thrombus burden increased dramatically from fully apposed (0.00 mm) to extremely malapposed (0.50 mm) from the lumen wall. Fully apposed stents had the least thrombus burden with 4.1 ± 0.5%, 0.10 mm malapposed stents had a thrombus burden of 6.3 ± 0.5%, 0.25 mm malapposed stents had a thrombus burden of 19.7 ± 4.5%, and finally, 0.50 mm malapposed stents had a thrombus burden of 92.6 ± 7.4% ($p < 0.0001$). (**C**) Blood from volunteers collected in 1% sodium citrates tubes was incubated for 15 min prior to perfusion through the stent thrombosis model. Bare-metal stents (BMS), drug-eluting stents (DES) and bioresorbable vascular scaffolds (BVS) were deployed into the model with malapposed distances of 0.25 mm from vessel wall. Volunteer blood samples were then perfused through the model for 15 min. BMS and DES showed similar levels of thrombus burden at 14.7 ± 3.8% and 20.5 ± 3.1%, respectively. BVS stents were significantly more thrombogenic than both BMS and DES, yielding thrombus burden of 86.8 ± 5.3% ($p < 0.01$). Same data was used for well- and mal-apposed stent segments from Figure 2A/B (1% citrate and 15 min) and Figure 3A. Data is expressed as mean±standard error to the mean and was analyzed by unpaired students t-test, ANOVA, and Tukey Test. $p < 0.05$ is considered statistically significant. ***$P < 0.001$. ****$P < 0.0001$.

device malapposition, we deployed stents of lesser diameter in our 3.0 mm tube. As the degree of malapposition increased relative to tubular lumen, thrombus burden increased exponentially, from well apposed (0.00 mm to vessel wall) to an extremely malapposed stent (0.50 mm from vessel wall). Well-apposed stents ($n = 6$) had the least thrombus burden with 4.1 ± 0.5%, 2.90 mm stents (0.10 mm malapposed from vessel wall) ($n = 4$) had a thrombus burden of 6.3 ± 0.5%, 2.75 mm stents (0.25 mm from vessel wall) ($n = 5$) had a thrombus burden of 19.7 ± 4.5%. Finally, extremely malapposed stents (0.50 mm from vessel wall) ($n = 4$) had a thrombus burden of 92.6 ± 7.4% ($p < 0.0001$, Figure 3B).

Finally, to determine the effect of stent design on thrombus formation, we deployed clinically available bare metal stents (BMS), drug-eluting stents (DES) and bioresorbable vascular scaffold (BVS) stents into the flow loops in a malapposed state and evaluated relative thrombogenicity (2.75 mm). As observed clinically, BVSs exhibited greater thrombus burden compared to both DES and BMS (86.8 ± 5.3%, 20.5 ± 3.1% and 14.7 ± 3.8% for BVS, DES, and BMS, respectively, $p < 0.01$, Figure 3C). In this model, BMS and DESs showed no differences in acute thrombus burden.

Evaluation of Clinically Validated Antiplatelet and Anticoagulant Regimens on Thrombus Burden

In order to examine the clinical applicability of our model we tested validated antithrombotic agents for prevention of thrombus formation either during intervention or following stent deployment. The following experiments were conducted using drug-eluting stents with malapposed sections under-expanded to 2.75 mm in diameter (0.25 mm from vessel wall). Control blood from healthy volunteers had a thrombus burden of 12.1 ± 1.8% ($n = 5$) and *in vitro* incubation with 20 μM ASA and 1.4 μM Ticagrelor reduced thrombus burden to 6.9 ± 1.7% ($n = 4$, $p = 0.02$). Blood incubated with DAPT had considerable reduction in platelet reactivity units (PRU) measured with VerifyNow from 183.3 ± 28.3 to 6.0 ± 2.6 PRU ($n = 3$) (**Supplemental Figure 3**). When volunteer blood was incubated with 1.0 IU/mL heparin, thrombus burden was reduced to 4.1 ± 0.6% ($n = 6$, $p < 0.001$). Target range of activated clotting time (ACT) is between 300 and 350 s and incubation with 1 IU/mL heparin increased ACT to 295.4 ± 16.3 s ($n = 3$) [16]. Incubation with 3.8 μM corn trypsin inhibitor reduced the thrombus burden to 2.5 ± 0.5% ($n = 3$, $p < 0.0001$). The combination of *in vitro* administration of DAPT and heparin resulted in a reduction of thrombus burden to 4.5 ± 1.2% ($n = 3$, $p < 0.01$). Finally, blood obtained from patients undergoing cardiac catheterization who received DAPT and intra-procedural heparin had a thrombus burden of 4.9 ± 0.4% ($n = 8$, $p < 0.001$) (Figure 4).

Discussion

The current study sought to demonstrate an *in vitro* stent thrombosis model as an efficient, rapid, and reliable method of accurately assessing thrombotic risk following coronary stenting. Herein, we describe the optimization and validation across a spectrum of pathology. Specifically, strut malapposition, antiplatelet and antithrombotic regimens, and device subtypes performed as expected given their associated clinical risk. As per previous findings, it has been demonstrated that differences in stent design – in particular strut thickness and polymer coating – contribute to the differences observed in acute thrombus generation as observed in our model [17]. Finally, antithrombotic agents reduced the risk of thrombus formation in our model as validated by numerous clinical trials [18–20]. Accordingly, this model addresses a need – enabling screening of novel device designs and/or for testing novel therapeutic targets for the prevention of ST.

To our knowledge, our model allows a systematic examination of the individual components of the pathophysiology responsible for ST using human blood which remains limited in animal models [21,22]. Largely due to the low incidence of stent thrombosis following PCI in both animal models and clinical studies, the associated risk of devices or therapeutic regimens requires unduly large studies to adequately assess risk. Currently, only a few additional models address this clinical need. First, a bench-top model which employs a modified Chandler loop has been reported using porcine blood but used qualitative visualization of washed segments to assess thrombosis – in contrast to our model which permits precise quantitative measurements by OCT [12]. Second, others have reported an *ex vivo* arteriovenous shunt model in swine to examine the effect of stent polymer and technology on acute stent thrombogenicity [17]. While an *ex vivo* swine model allows flow conditions similar to what is observed clinically, it requires sacrifice of the animal and does not permit ease of testing therapeutic targets. Thus, our model affords the distinct

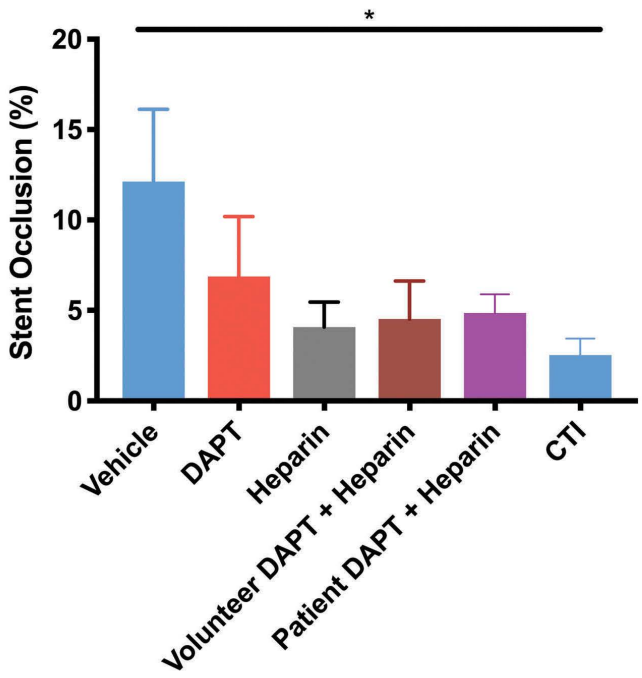


Figure 4. **Effect of antithrombotic agents on thrombus burden in the context of stent malapposition**. 1% citrated blood from volunteers was incubated with 20.0 μM ASA and 1.4 μM Ticagrelor (DAPT), 1 IU/mL of heparin, combination of DAPT+heparin, 3.8 μM corn trypsin inhibitor (CTI), or DMSO vehicle control for 15 min prior to perfusion through the Endeavor-RX drug-eluting stent. Patient samples obtained for analysis had a loading dose of $P2Y_{12}$ inhibitor and ASA and IV infusion of heparin during coronary catheterization. These samples were perfused for 15 min through the bench-top stent thrombosis model. Vehicle (DMSO) control from volunteers had a thrombus burden of 12.1 $\pm$ 1.8% and incubation with DAPT had a reduced thrombus burden to 6.9 $\pm$ 1.7%. When the same blood was incubated with heparin, thrombus burden was reduced to 4.1 $\pm$ 0.6%. Blood incubated with DAPT+Heparin had a thrombus burden of 4.5 $\pm$ 1.2%. Blood obtained from patients had a thrombus burden of 4.9 $\pm$ 0.4% ($p < 0.01$). Finally, blood incubated with 3.8 μM CTI had a thrombus burden of 2.5 $\pm$ 0.5%. Data is expressed as mean$\pm$standard error to the mean and was analyzed by ANOVA and Tukey Test. $p \leq 0.05$ is considered statistically significant.

advantage of testing human samples, across a spectrum of device parameters all while providing quantitative data not afforded in previously described models.

Importantly, our work highlights the reproducibility of our model with previous findings regarding device apposition and design characteristics. In particular, our model demonstrated incremental increase in thrombus burden associated with increasing degree of stent underdeployment similar to previous findings [11,12,23]. Secondarily, our work emphasizes the importance of polymer material and strut thickness in optimizing stent technology. Indeed, we were able to demonstrate a larger in-stent thrombus burden when blood was circulated through BVS compared to DES or BMS. Both ABSORB II and III trials have demonstrated an increased rate of stent or device thrombosis in BVS compared to DES [24,25]. Our model found in-stent thrombus burden was comparable for DES and BMS – similar to short-term outcomes in stents of comparable strut thickness and design. Thus, our model may serve as a screen for thrombogenic risk during the design phase of novel intra-coronary devices or coatings moving forward informing relative risk prior to moving to large animal or early clinical studies.

Finally, by employing human samples in our model we are able to both test the therapeutic efficacy of administered therapies as well as screen novel therapies for efficacy. Specifically, thrombus burden was reduced after both exogenous administration of anti-platelet regimens as well as when regimens were administered to patients prior to blood collection. Similarly, novel therapeutics could be screened and tested by secondary administration *or* efficacy could be evaluated in early human studies by collecting blood from on-treatment patients. While the use of platelet function testing has failed to change clinical outcomes in trials, our model has the added advantage of testing the interface under flow parameters that mimic that in a human coronary while interacting with an "at-risk" device. Thus, the low cost, rapidity and fidelity of the system all lend itself to implementation early in the therapeutic development process.

Certainly, our study is not without its limitations. First, the model does not capture the entirety of the complex pathophysiologic environment observed *in vivo* – particular the role of the vessel as an activator and mediator of the thrombotic milieu. Thus, the role of inflammation, endothelial dysfunction and underlying atherosclerosis are not adequately evaluated; however, outside of clinical studies animal models of ST are exclusively performed in normal arteries. Second, our system requires baseline anticoagulation to prevent sample thrombosis prior to circulation. We elected to use citrate to bind calcium and minimize the impact on platelet/thrombotic function. Thus, it is possible that subtle but important difference could be missed at lower levels of anticoagulation. However, the reported system provides the most quantitative and promising model to study the effect of novel antithrombotic agents or device design for their impact on stent thrombosis in a rapid and economical manner. Third, our model is unable to evaluate complex calcified atherosclerosis which creates irregular arteries potentially contributing to stent malapposition or bifurcation lesions which are often sites of atherosclerotic lesions. Future experiments will aim to study the role of these complex lesions by incorporating modern 3D printing models [26].

Conclusion

Evaluation of *in situ* thrombus formation in a bench-top model of ST demonstrates device and drug performance comparable to clinical findings. This rapid and cost-effective system appears promising to screen novel pharmaceutical targets and device designs prior to clinical testing.

Abbreviations

ACS acute coronary syndrome

ASA	acetylsalicylic acid
BMS	bare metal stent
BVS	bioresorbable vascular scaffold
CAD	coronary artery disease
DAPT	dual antiplatelet therapy
DES	drug eluting stent
OCT	optical coherence tomography
PCI	percutaneous coronary intervention
ST	stent thrombosis

Funding

This project was supported by the Innovation Fund of the Alternative Funding Plan for the Academic Health Sciences Centres of Ontario and Canadian Foundation of Innovation. R.G.J. was funded by the Vanier CIHR Canada Graduate Scholarship for his graduate studies. A.C.D. was supported by the Cardiac Endowment Fund at the University of Ottawa Heart Institute.

Disclosure Statement

The authors declare that they have no competing interests.

Supplementry material

Supplemental data for this article can be accessed on the publisher's website.

Authors' contributions

R.G.J., D.P.N., N.F., D.S., B.H. participated in the design, data extraction and analysis, and drafted the manuscript. A.L., O.D., R. L., F.D.R., P.D.S., and D.K. participated in data collection and analysis. A.C.D., T.S., K.M., K.S., and R.R. participated in data analysis. All authors were involved in the critical revision and approved the final draft of the manuscript.

References

1. Simard T, Hibbert B, Ramirez FD, Froeschl M, Chen YX, O'Brien ER. The evolution of coronary stents: A brief review. Can J Cardiol 2014;30(1):35–45. doi:10.1016/j.cjca.2013.09.012.
2. Stettler C, Wandel S, Allemann S, Kastrati A, Morice MC, Schomig A, Pfisterer ME, Stone GW, Leon MB, de Lezo JS, et al. Outcomes associated with drug-eluting and bare-metal stents: A collaborative network meta-analysis. Lancet 2007;370 (9591):937–948. doi:10.1016/S0140-6736(07)61444-5.
3. Byrne RA, Joner M, Kastrati A. Stent thrombosis and restenosis: what have we learned and where are we going? The andreas grüntzig lecture esc 2014. Eur Heart J 2015;36(47):3320–3331. doi:10.1093/eurheartj/ehv511.
4. Tada T, Byrne RA, Simunovic I, King LA, Cassese S, Joner M, Fusaro M, Schneider S, Schulz S, Ibrahim T et al. Risk of stent thrombosis among bare-metal stents. First-Generation Drug-Eluting Stents, and Second-Generation Drug-Eluting Stents 2013;6 (12):1267–1274.
5. Yusuf S, Zhao F, Mehta SR, Chrolavicius S, Tognoni G, Fox KK, Clopidogrel in Unstable Angina to Prevent Recurrent Events Trial I. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without st-segment elevation. N Engl J Med 2001;345(7):494–502. doi:10.1056/NEJMoa010746.
6. Mehta SR, Yusuf S, Peters RJG, Bertrand ME, Lewis BS, Natarajan MK, Malmberg K, Rupprecht H-J, Zhao F, Chrolavicius S, et al. Effects of pretreatment with clopidogrel and aspirin followed by long-term therapy in patients undergoing percutaneous coronary intervention: the pci-cure study. The Lancet 2001;358(9281):527–533. doi:10.1016/S0140-6736(01)05701-4.
7. Cutlip DE, Windecker S, Mehran R, Boam A, Cohen DJ, van Es GA, Steg PG, Morel MA, Mauri L, Vranckx P, et al. Clinical end points in coronary stent trials: A case for standardized definitions. Circulation 2007;115(17):2344–2351. doi:10.1161/CIRCULATIONAHA.106.685313.
8. Windecker S, Meier B. Late coronary stent thrombosis. Circulation 2007;116(17):1952–1965. doi:10.1161/CIRCULATIONAHA.106.683995.
9. Bezerra HG, Costa MA, Guagliumi G, Rollins AM, Simon DI. Intracoronary optical coherence tomography: A comprehensive review: clinical and research applications. JACC Cardiovasc Interv 2009;2(11):1035–1046. doi:10.1016/j.jcin.2009.06.019.
10. Cook S, Wenaweser P, Togni M, Billinger M, Morger C, Seiler C, Vogel R, Hess O, Meier B, Windecker S. Incomplete stent apposition and very late stent thrombosis after drug-eluting stent implantation. Circulation 2007;115(18):2426–2434. doi:10.1161/CIRCULATIONAHA.106.658237.
11. Foin N, Gutierrez-Chico JL, Nakatani S, Torii R, Bourantas CV, Sen S, Nijjer S, Petraco R, Kousera C, Ghione M, et al. Incomplete stent apposition causes high shear flow disturbances and delay in neointimal coverage as a function of strut to wall detachment distance: implications for the management of incomplete stent apposition. Circ Cardiovasc Interv 2014;7(2):180–189. doi:10.1161/CIRCINTERVENTIONS.113.000931.
12. Kolandaivelu K, Swaminathan R, Gibson WJ, Kolachalama VB, Nguyen-Ehrenreich KL, Giddings VL, Coleman L, Wong GK, Edelman ER. Stent thrombogenicity early in high-risk interventional settings is driven by stent design and deployment and protected by polymer-drug coatings. Circulation 2011;123(13):1400–1409. doi:10.1161/CIRCULATIONAHA.110.003210.
13. Kubo T, Akasaka T, Shite J, Suzuki T, Uemura S, Yu B, Kozuma K, Kitabata H, Shinke T, Habara M, et al. Oct compared with ivus in a coronary lesion assessment: the opus-class study. JACC Cardiovasc Imaging 2013;6(10):1095–1104. doi:10.1016/j.jcmg.2013.04.014.
14. Ali ZA, Maehara A, Genereux P, Shlofmitz RA, Fabbiocchi F, Nazif TM, Guagliumi G, Meraj PM, Alfonso F, Samady H, et al. Optical coherence tomography compared with intravascular ultrasound and with angiography to guide coronary stent implantation (ilumien iii: optimize pci): A randomised controlled trial. Lancet 2016;388(10060):2618–2628. doi:10.1016/S0140-6736(16)31922-5.
15. Tearney GJ, Regar E, Akasaka T, Adriaenssens T, Barlis P, Bezerra HG, Bouma B, Bruining N, Cho JM, Chowdhary S, et al. Consensus standards for acquisition, measurement, and reporting of intravascular optical coherence tomography studies: A report from the international working group for intravascular optical coherence tomography standardization and validation. J Am Coll Cardiol 2012;59(12):1058–1072. doi:10.1016/j.jacc.2011.09.079.
16. Montalescot G, White HD, Gallo R, Cohen M, Steg PG, Aylward PEG, Bode C, Chiariello M, King SB, Harrington RA et al. Enoxaparin versus unfractionated heparin in elective percutaneous coronary intervention. N Engl J Med 2006;355(10):1006–1017. doi:10.1056/NEJMoa052711.
17. Otsuka F, Cheng Q, Yahagi K, Acampado E, Sheehy A, Yazdani SK, Sakakura K, Euller K, Perkins LEL, Kolodgie FD, et al. Acute thrombogenicity of a durable polymer everolimus-eluting stent relative to contemporary drug-eluting stents with biodegradable polymer coatings assessed ex vivo in a swine shunt model. JACC Cardiovasc Interv 2015;8(9):1248–1260. doi:10.1016/j.jcin.2015.03.029.
18. Bonaca MP, Bhatt DL, Cohen M, Steg PG, Storey RF, Jensen EC, Magnani G, Bansilal S, Fish MP, Im K, et al. Long-term use of ticagrelor in patients with prior myocardial infarction. N Engl J Med 2015;372(19):1791–1800. doi:10.1056/NEJMoa1500857.
19. Mauri L, Kereiakes DJ, Yeh RW, Driscoll-Shempp P, Cutlip DE, Steg PG, Normand S-LT, Braunwald E, Wiviott SD, Cohen DJ, et al. Twelve or 30 months of dual antiplatelet therapy after drug-eluting stents. N Engl J Med 2014;371(23):2155–2166. doi:10.1056/NEJMoa1409312.
20. Stone GW, Witzenbichler B, Guagliumi G, Peruga JZ, Brodie BR, Dudek D, Kornowski R, Hartmann F, Gersh BJ, Pocock SJ, et al. Bivalirudin during primary pci in acute myocardial infarction. N Engl J Med 2008;358(21):2218–2230. doi:10.1056/NEJMoa0708191.
21. Schwartz RS, Edelman ER, Carter A, Chronos NA, Rogers C, Robinson KA, Waksman R, Machan L, Weinberger J, Wilensky RL, et al. Preclinical evaluation of drug-eluting stents for peripheral applications: recommendations from an expert consensus group. Circulation 2004;110(16):2498–2505. doi:10.1161/01.CIR.0000145164.85178.2E.
22. Jeong MH, Owen WG, Staab ME, Srivatsa SS, Sangiorgi G, Stewart M, Holmes DR Jr., Schwartz RS. Porcine model of stent thrombosis: platelets are the primary component of acute stent

closure. Cathet Cardiovasc Diagn 1996;38(1):38–43. doi:10.1002/(SICI)1097-0304(199605)38:1<38::AID-CCD9>3.0.CO;2-4.
23. Attizzani GF, Capodanno D, Ohno Y, Tamburino C. Mechanisms, pathophysiology, and clinical aspects of incomplete stent apposition. J Am Coll Cardiol 2014;63(14):1355–1367. doi:10.1016/j.jacc.2014.01.019.
24. Kereiakes DJ, Ellis SG, Metzger C, Caputo RP, Rizik DG, Teirstein PS, Litt MR, Kini A, Kabour A, Marx SO, et al. 3-year clinical outcomes with everolimus-eluting bioresorbable coronary scaffolds: the absorb iii trial. J Am Coll Cardiol 2017;70(23):2852–2862. doi:10.1016/j.jacc.2017.10.010.
25. Serruys PW, Chevalier B, Sotomi Y, Cequier A, Carrié D, Piek JJ, Van Boven AJ, Dominici M, Dudek D, McClean D, et al. Comparison of an everolimus-eluting bioresorbable scaffold with an everolimus-eluting metallic stent for the treatment of coronary artery stenosis (absorb ii): A 3 year, randomised, controlled, single-blind, multicentre clinical trial. The Lancet 2016;388(10059):2479–2491. doi:10.1016/S0140-6736(16)32050-5.
26. Garcia J, Yang Z, Mongrain R, Leask RL, Lachapelle K. 3d printing materials and their use in medical education: A review of current technology and trends for the future. BMJ Simul Technol Enhanc Learn 2018;4(1):27. doi:10.1136/bmjstel-2017-000234.

Appendix P

Photoplethysmography using a smartphone application for assessment of ulnar artery patency: a randomized clinical trial

Citation information:

Di Santo P**,** Harnett DT, Simard T, Ramirez FD, Pourdjabbar A, Yousef A, Moreland R, Bernick J, Wells GA, Dick A, Le May M, Labinaz M, So D, Motazedian P, **Jung RG**, Chandrashekar J, Mehran R, Chong AY, Hibbert B. Photoplethysmography using a smartphone application for assessment of ulnar artery patency: a randomized clinical trial. **(2018)**. *CMAJ.* 190(13):E380-E388

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RESEARCH

Photoplethysmography using a smartphone application for assessment of ulnar artery patency: a randomized clinical trial

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Cite as: *CMAJ* 2018 April 3;190:E380-8. doi: 10.1503/cmaj.170432

See related article at www.cmaj.ca/lookup/doi/10.1503/cmaj.180269

ABSTRACT

BACKGROUND: Radial artery access is commonly performed for coronary angiography and invasive hemodynamic monitoring. Despite limitations in diagnostic accuracy, the modified Allen test (manual occlusion of radial and ulnar arteries followed by release of the latter and assessment of palmar blush) is used routinely to evaluate the collateral circulation to the hand and, therefore, to determine patient eligibility for radial artery access. We sought to evaluate whether a smartphone application may provide a superior alternative to the modified Allen test.

METHODS: We compared the modified Allen test with a smartphone heart rate–monitoring application (photoplethysmography readings detected using a smartphone camera lens placed on the patient's index finger) in patients undergoing a planned cardiac catheterization. Test order was randomly assigned in a 1:1 fashion. All patients then underwent conventional plethysmography of the index finger, followed by Doppler ultrasonography of the radial and ulnar arteries (the diagnostic standard). The primary outcome was diagnostic accuracy of the heart rate–monitoring application.

RESULTS: Among 438 patients who were included in the study, we found that the heart rate–monitoring application had a superior diagnostic accuracy compared with the modified Allen test (91.8% v. 81.7%, $p = 0.002$), attributable to its greater specificity (93.0% v. 82.8%, $p = 0.001$). We also found that this application had greater diagnostic accuracy for assessment of radial or ulnar artery patency in the ipsilateral and contralateral wrist (94.0% v. 84.0%, $p < 0.001$).

INTERPRETATION: A smartphone application used at the bedside was diagnostically superior to traditional physical examination for confirming ulnar patency before radial artery access. This study highlights the potential for smartphone-based diagnostics to aid in clinical decision-making at the patient's bedside. **Trial registration:** Clinicaltrials.gov, no. NCT02519491.

The radial artery is used commonly for coronary angiography, percutaneous coronary intervention and invasive hemodynamic monitoring. Transradial access for coronary and peripheral interventions is used increasingly because of a lower incidence of vascular complications, patient preference and reduced mortality in patients with acute coronary syndrome.[1–4] Many of these benefits are attributed to superior postprocedural hemostasis given the superficial course and small calibre of the radial artery relative to the deeper and larger femoral vessel. However, this small calibre also renders the radial artery prone to occlusion if proper anticoagulation and hemostatic practices are not followed[5,6] — a complication that is reported to occur in up to one-third of patients.[7] Despite dual blood supply to the hand via the ulnar and radial arteries, rare cases of substantial limb compromise have been reported after catheterization of the radial artery.[8–10] Evaluating the competency of this dual blood supply before radial artery instrumentation continues to be performed routinely,[11] despite questions raised about its value,[12] and it

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remains endorsed by current interventional cardiac societies,[13,14] as well as the World Health Organization.[15]

Colour Doppler ultrasonography imaging is the gold standard method for assessing arterial patency and collateral competency in this setting,[16] but it is relatively resource-intensive and, therefore, seldom feasible or cost-effective in routine clinical practice. Assessment at the bedside using the modified Allen test is widely accepted as the clinical standard. Despite this test's known diagnostic and prognostic shortcomings[16,17] and the low incidence of clinically significant ulnar artery occlusion,[18] results from modified Allen tests have served to exclude as many as 27% of patients from transradial approaches for cardiac catheterization, and an abnormal modified Allen test remains a relative contraindication for placement of invasive hemodynamic monitoring.[19] Plethysmography, which is the measurement of changes in amplitude of a photoelectric signal based on changes in blood volume pulsations,[20] and pulse oximetry (commonly referred to as the Barbeau test) have been described as alternatives; however, the need for a dedicated oximeter has precluded its widespread use. Therefore, the modified Allen test remains the preferred method for assessment of dual circulation.[11,21]

Because of the widespread availability of smartphones, they are being used increasingly as point-of-care diagnostics in clinical settings with minimal or no cost. For example, built-in cameras with dedicated software or photodiode sensors using infrared light–emitting diodes have the potential to render smartphones into functional plethysmographs. However, robust evaluation of these technologies in clinical settings is lacking. We sought to evaluate the diagnostic accuracy of a smartphone-based heart rate–monitoring application compared with the modified Allen test for assessing dual circulation to the hand (ulnar artery patency) for transradial angiography.

Methods

Study design and participants

The CAPITAL iPhone-based evaluation of dual circulation before transRadial Access for Diagnostic Angiography triaL (CAPITAL iRADIAL) was a prospective randomized clinical trial (RCT) conducted at the University of Ottawa Heart Institute between July 2015 and March 2016. We recruited patients from the hospital's coronary care unit, inpatient cardiology service and Regional Referral Centre/Day Unit if their clinical care plan included assessment for cardiac catheterization that included possible transradial access (Figure 1). Patients with a planned cardiac catheterization were eligible for inclusion. We excluded patients if hemodynamic instability was present, there was a need for emergent angiography (ongoing cardiac ischemia or ST-segment elevation myocardial infarction), the patient could not or would not provide informed consent or the patient had undergone previous surgical removal of the radial or ulnar artery. All participants provided written informed consent.

Randomization and masking

We randomly assigned participants 1:1 to either the modified Allen test or heart rate–monitoring application by using concealed envelopes prepared with a computer random sequence generator. Either the modified Allen test or heart rate–monitoring application were performed as the initial assessment based on group allocation. Subsequently, all participants underwent 3 additional assessments: either a modified Allen test or heart rate–monitoring application (whichever was not performed first), followed by plethysmography (Barbeau and reverse Barbeau) and Doppler ultrasonography of the radial and ulnar arteries. All assessments were conducted by the same co-investigator, and, therefore, blinding was not performed.

The modified Allen test was performed on all participants by having the patient make a fist for 30 seconds and with pressure applied over the radial and ulnar arteries to occlude them. The participant was then instructed to open the fist, and the ulnar artery was selectively released while the co-investigator monitored for return of colour to the hand (Figure 2, supplementary video 1 [Appendix 1, available at www.cmaj.ca/lookup/suppl/doi:10.1503/cmaj.170432/-/DC1]). We coded the result as either ulnar artery patent (maximal palmar blush ≤ 5 s) or occluded (maximal palmar blush occurring > 5 s). This technique was then repeated by maintaining compression over the ulnar artery and releasing the compression over the radial artery ("reverse modified Allen test") and using the same coding criteria. A 5-second cut-off for arterial patency was chosen because it has been shown to maximize diagnostic accuracy (79.6%), with a sensitivity of 75.8% and specificity of 81.7%.[16]

We conducted the smartphone-based evaluation by using the Instant Heart Rate application (version 4.5.0; Azumio) on an iPhone 4S (Apple). We selected this application because it permits direct visualization of the photoplethysmography tracing on the screen, is commercially available free of charge on both Apple and Android operating systems and is the most downloaded heart rate–monitoring application worldwide. The application software was initially developed for tracking heart rate in individuals without the need for dedicated equipment, using a proprietary algorithm by Azumio that was validated by comparing data collected via the application to electrocardiograms. The software utilizes the smartphone's camera light and lens to reflect light on an individual's finger, resulting in colour and brightness changes that are then extracted by the camera lens and interpreted as changes in pulse, reflected both as a numerical value (i.e., heart rate) and as a photoplethysmography tracing on the phone's screen.

We acquired photoplethysmography readings by placing the camera lens over the pulp of the participant's index finger with the hand resting on a firm surface and light pressure applied over the back of the camera (Figure 2, supplementary video 2 [Appendix 2, available at www.cmaj.ca/lookup/suppl/doi:10.1503/cmaj.170432/-/DC2]). Readings were recorded before and immediately after isolated radial artery compression for 2 minutes or less and were divided into 4 categories as previously described by Barbeau and colleagues[21] (i.e., A, no damping of pulse tracing immediately after radial artery compression; B, damping of pulse tracing; C, loss of pulse tracing followed by recovery of pulse tracing within 2 minutes; D, loss of pulse tracing without recovery within 2 minutes). Readings categorized as A or B indicated

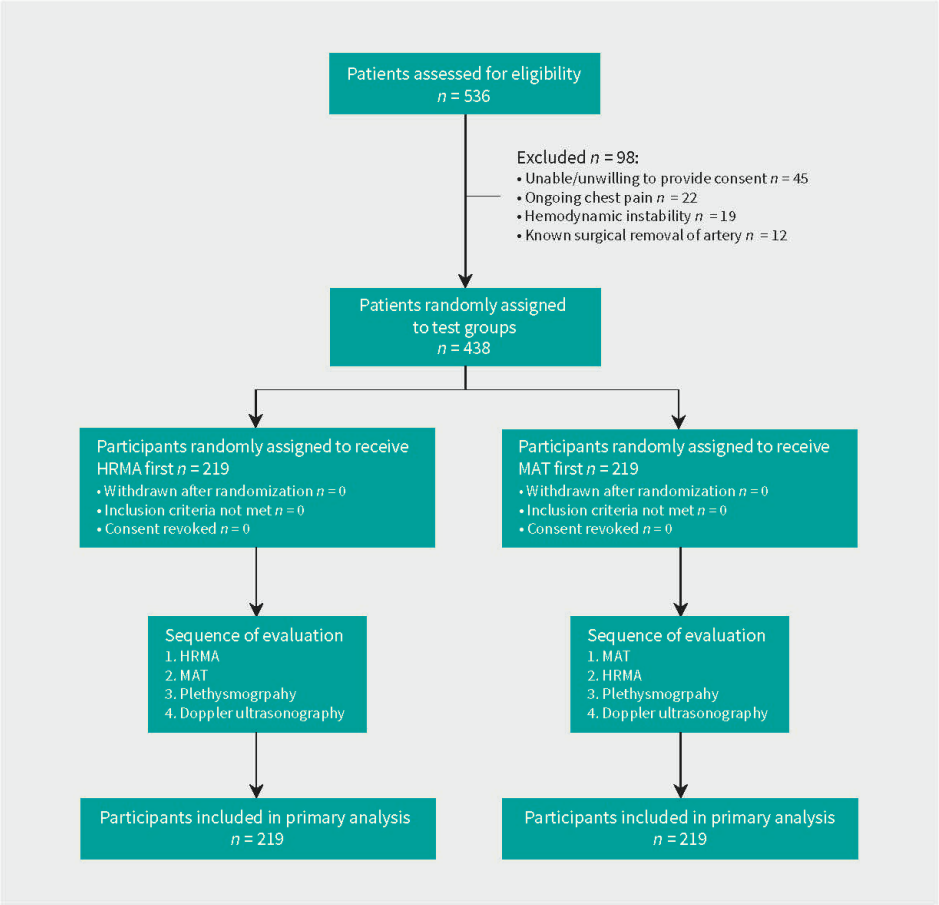


Figure 1: Participant recruitment and allocation. HRMA = heart rate–monitoring application, MAT = modified Allen test.

adequate flow, whereas those characterized as C or D indicated inadequate circulation.

We used a portable plethysmograph and pulse oximeter (Summit DME) for all participants after the assessments using the modified Allen test and heart rate–monitoring application, and before the assessments conducted with Doppler ultrasonography. We classified readings from plethysmography by using the same criteria as for the heart rate–monitoring application. We performed Doppler ultrasonography by using a linear transducer probe at both left and right wrists (Hitachi Noblus). We used both short and long axis views to visualize and measure radial and ulnar arteries. We measured maximum lumen diameter, intimal thickness and colour Doppler with pulse wave velocities at 1 cm proximal to the styloid process of the radius.[22]

Outcomes

Our primary outcome was overall diagnostic accuracy (the total number of true positives and true negatives as a proportion of the entire sample size) of the heart rate–monitoring application or modified Allen test for assessing collateral blood supply to the hand. For the primary analysis, we used only the first test to which the patient was randomly assigned to evaluate diagnostic accuracy, and then we compared test accuracy between the 2 randomly asssigned groups. We used the complete absence of any detectable Doppler flow of the ulnar artery as the reference standard for a positive modified Allen test or heart rate–monitoring application. We used the diagnostic accuracy of the heart rate–monitoring application for radial or ulnar artery patency from both right and left wrists (irrespective of expected primary access site) as a secondary outcome.

We evaluated prespecified variables to determine the predictors of poor diagnostic performance for the heart rate–monitoring application. We also compared the diagnostic accuracy of the application to that of conventional plethysmography. In addition, we conducted a post hoc analysis to determine if a stepwise approach to dual circulation assessment, specifically performing

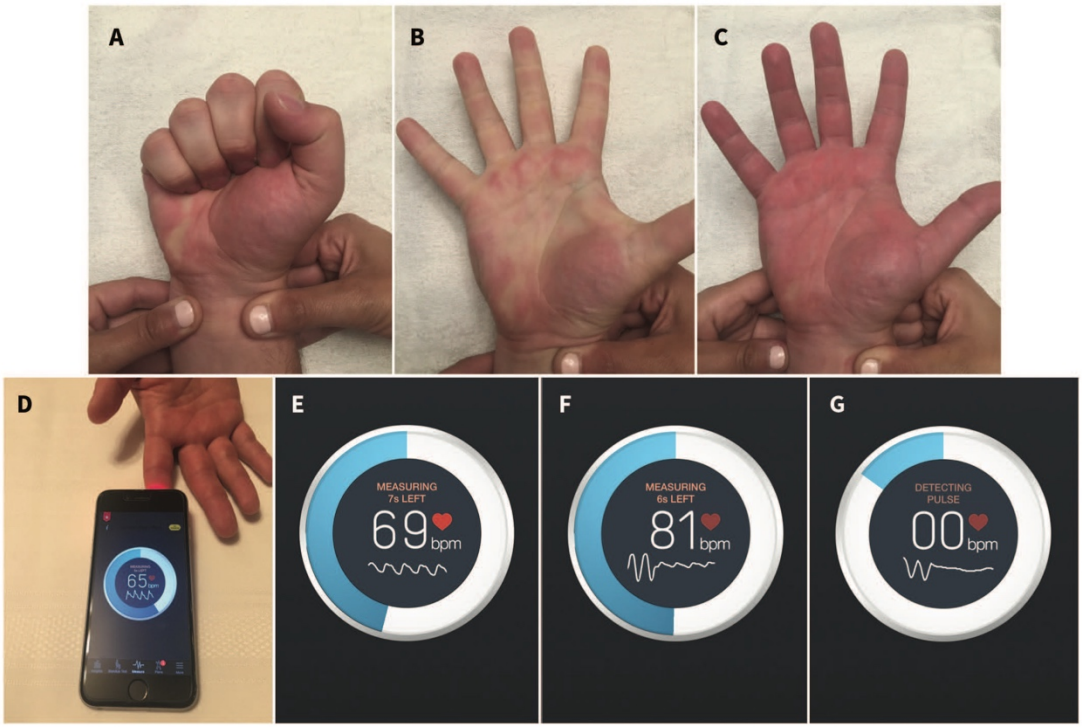


Figure 2: Demonstration of the modified Allen test and the smartphone-based heart rate–monitoring application. (A) Bilateral occlusion of the radial and ulnar arteries followed by clenching and opening of the hand 3 times. (B) Release of compression of the ulnar artery with blanching of the hand. (C) Restoration of palmar blush within 5 seconds, suggesting patency of the ulnar artery. (D) Placement of the smartphone camera on the index finger. (E) Screenshot capture of the instant heart rate interface showing a normal photoplethysmographic tracing below the participant's heart rate with isolated radial artery compression. (F) Dampening of the pulse tracing with isolated radial artery compression. (G) Loss of the pulse tracing with radial artery compression. Note: bpm = beats per minute.

the modified Allen test followed by the use of the heart rate–monitoring application in cases of positive modified Allen test results, resulted in greater diagnostic accuracy compared to the modified Allen test alone.

Statistical analysis

We assumed a diagnostic accuracy of 80% using the modified Allen test,[16] and 438 participants were calculated to achieve 80% power to detect a 10% difference in diagnostic accuracy using the heart rate–monitoring application. All continuous variables are reported as means (standard deviation). Categorical variables are described as number (%). Comparisons between diagnostic arms were performed using χ^2 or Fisher exact tests. Within arm comparisons were performed using McNemar tests when comparing proportions for paired data. We analyzed artery-level data by using generalized estimating equations. We took clustering into account for those participants who had multiple arteries assessed. We used logistic regression to generate the model for predictors of false-positive assessment. All calculations were performed using SAS version 9.4 (SAS Institute). Our research protocol can be found in Appendix 3 (available at www.cmaj.ca/lookup/suppl/doi:10.1503/cmaj.170432/-/DC3). The study was registered with clinicaltrials.gov (NCT02519491).

Ethics approval

The study was approved by the Ottawa Health Science Network Research Ethics Board.

Results

A total of 536 patients were screened, of whom 438 were included (Figure 1). Patient characteristics were comparable between the modified Allen test and heart rate monitoring application arms (Table 1). Nearly half of the participants in each group had previously undergone coronary angiography or had a documented history of arterial line insertion. Medical therapy, including nitrates and anticoagulants, was similar between the groups. The right radial artery was the potential primary access site in 75.8% of participants, and maximal vessel and lumen diameters were similar between the 2 groups. The median baseline lumen diameter was 2.1 (interquartile range 1.8–2.5) mm, and comparable proportions of patients within each Barbeau class were randomly assigned to the arms.

Primary outcome: ulnar artery patency assessment

Absence of ulnar flow by Doppler ultrasonography in the homolateral limb was found in 10 participants (6 in the heart rate–monitoring application group and 4 in the modified Allen test group). Diagnostic accuracy was 10.1% higher using the heart rate–monitoring application compared to that with the modified Allen test for determining ulnar artery patency (91.8%, 95% confidence interval [CI] 87.3% to 95.1% v. 81.7%, 95% CI 76.0% to 86.6%, $p = 0.002$, Table 2). This was caused by greater specificity with the heart rate–monitoring application (93.0%, 95% CI 88.7% to 96.0% v. 82.8%, 95% CI 77.1% to 87.6%, $p = 0.001$). On a per-participant basis, 15 participants (6.8%) in the heart rate–monitoring application group and 37 (16.9%) in the modified Allen test group had false-positive results and would have been incorrectly excluded from radial artery access. Neither sensitivity (50.0%, 95% CI 11.8% to 88.2% v. 25.0%, 95% CI 0.6 to 80.6, $p = 0.6$) nor positive predictive value differed between the heart rate–monitoring application and the modified Allen test groups (16.7%, 95% CI 3.6% to 41.4% v. 2.6%, 95% CI 0.7% to 13.8%, $p = 0.09$). The negative predictive value was 98.3% (95% CI 95.2% to 99.7%) for the modified Allen test group compared to 98.5% (95% CI 95.7% to 99.7%) in the heart rate–monitoring application group ($p = 1.0$).

Dual circulation assessment in all arteries

In total, 1472 arteries were assessed using the modified Allen test, heart rate–monitoring application, plethysmography and Doppler ultrasonography in both homolateral and contralateral limbs (Table 3). The discriminatory power of the heart rate–monitoring application remained greater than that of the modified Allen test, with a diagnostic accuracy of 94.0% (95% CI 91.9% to 95.6%) compared to 84.0% (95% CI 79.9% to 97.4%), respectively ($p < 0.001$). We did not observe any differences in diagnostic performance of the modified Allen test or heart rate–monitoring application when we compared radial and ulnar assessments ($p > 0.05$).

Predictors of false-positive assessments

We included age, sex, body mass index (BMI) and presence of a palpable pulse as variables in the model. Thirty-seven participants in the modified Allen test cohort and 15 in the heart rate–monitoring application cohort had false-positive assessments. In the modified Allen test cohort, we found that age, male sex and BMI were not significant; however, the presence of a palpable ulnar artery significantly decreased the odds of having a false-positive result (odds ratio [OR] 0.06, 95% CI 0.02 to 0.25). Similarly, for the heart rate–monitoring application cohort, the only significant variable in the model was the presence of a palpable pulse (OR 0.04, 95% CI 0.01 to 0.15).

Table 1: Participant characteristics

Characteristic	No. (%) of participants, modified Allen test† $n = 219$	No. (%) of participants, heart rate–monitoring application† $n = 219$
Sex, male	136 (62.1)	149 (68.0)
Age, yr; mean ± SD	65.6 ± 13.2	67.0 ± 12.5
Height, m; mean ± SD	1.69 ± 0.1	1.70 ± 0.1
Weight, kg; mean ± SD	83.4 ± 21.1	84.3 ± 20.2
Body mass index, kg/m^2; mean ± SD	28.9 ± 6.3	29.0 ± 6.4
Heart rate, bpm; mean ± SD	71.5 ± 15.2	71.2 ± 14.2
Ethnic origin		
White	199 (90.9)	208 (95.0)
Nonwhite	20 (9.1)	11 (5.0)
Medical history		
Hypertension	144 (65.8)	149 (68.0)
Diabetes	90 (41.1)	78 (35.6)
Smoking	59 (26.9)	67 (30.6)
Family history of premature coronary artery disease*	48 (21.9)	50 (22.8)
Dyslipidemia	135 (61.6)	151 (68.9)
Previous myocardial infarction	101 (46.1)	105 (47.9)
Previous angiography	109 (49.8)	107 (48.9)
Previous percutaneous coronary intervention	76 (34.7)	72 (32.9)
Previous coronary artery bypass graft	42 (19.2)	38 (17.4)
Previous stroke	20 (9.1)	13 (5.9)
Previous arterial line	29 (13.2)	27 (12.3)
Medications		
Angiotensin-converting enzyme inhibitor/ angiotensin receptor blocker	108 (49.3)	112 (51.1)
β-Blocker	169 (77.2)	149 (68.0)
Statin	161 (73.5)	167 (76.3)
Acetylsalicylic acid	162 (74.0)	168 (76.7)
Clopidogrel/ticagrelor	98 (44.7)	91 (41.6)
Warfarin	23 (10.5)	26 (11.9)
Direct oral anticoagulant	6 (2.7)	18 (8.2)
Unfractionated heparin	25 (11.4)	24 (11.0)
Low-molecular-weight heparin	25 (11.4)	15 (6.8)
Bivalirudin	1 (0.5)	1 (0.5)
Nitrate	33 (15.1)	30 (13.7)

Note: bpm = beats per minute, SD = standard deviation.
*Defined as having a first-degree relative with clinically overt coronary artery disease (myocardial infarction, angina pectoris, need for coronary artery revascularization) before age 55 years (men) or 65 years (women).
†Unless otherwise specified.

Table 2: Diagnostic accuracy of the modified Allen test and the heart rate–monitoring application for assessing potential primary access sites

Parameter	No. (%) of participants,‡ modified Allen test *n* = 219	No. (%) of participants,‡ heart rate–monitoring application *n* = 219	*p* value§
Positive test result*	38 (17.4)	18 (8.2)	0.008
Overall diagnostic accuracy, % (95% CI)	81.7 (76.0 to 86.6)	91.8 (87.3 to 95.1)	0.002
Specificity, % (95% CI)	82.8 (77.1 to 87.6)	93.0 (88.7 to 96.0)	0.001
Sensitivity, % (95% CI)	25.0 (0.6 to 80.6)	50.0 (11.8 to 88.2)	0.6
Negative predictive value, % (95% CI)	98.3 (95.2 to 99.7)	98.5 (95.7 to 99.7)	1.0
Positive predictive value, % (95% CI)	2.6 (0.7v13.8)	16.7 (3.6 to 41.4)	0.09
Positive likelihood ratio (95% CI)	1.5 (–1.1 to 4.0)	7.1 (0.4 to 13.8)	
Negative likelihood ratio (95% CI)	0.9 (0.4 to 1.4)	0.5 (0.1 to 1.0)	
Barbeau classification†			
Class A	123 (56.2)	111 (50.7)	0.3
Class B	79 (36.1)	93 (42.5)	0.2
Class C	16 (7.3)	13 (5.9)	0.5
Class D	1 (0.5)	2 (0.9)	0.6

Note: CI = confidence interval.
*A positive result indicates that there is inadequate collateral blood flow; this includes all true-positive and false-positive results.
†Barbeau classification was based on conventional plethysmography results: class A, no damping of pulse tracing immediately after radial artery compression; class B, damping of pulse tracing; class C, loss of pulse tracing followed by recovery of pulse tracing within 2 minutes; class D, loss of pulse tracing without recovery within 2 minutes.
‡Unless specified otherwise.
§Values of *p* were generated using the Fisher exact test for between-arm comparisons.

Stepwise evaluation of dual circulation

We evaluated the clinical utility of sequential testing by determining the impact of the heart rate–monitoring application on participants who had a positive modified Allen test (i.e., palmar blush > 5 s). Of the patients randomly assigned to the modified Allen test cohort for our primary analysis, 38 (17.4%) participants had a positive modified Allen test and would be excluded from radial instrumentation. Of these participants, 36 had a subsequent normal heart rate–monitoring application assessment, thereby reclassifying these participants as having a patent ulnar artery. When compared with the use of the modified Allen test alone, this stepwise approach had a superior diagnostic accuracy (95.4%, 95% CI 91.8%–97.8% v. 81.7%, 95% CI 76.0%–86.6%, $p < 0.001$). This improved accuracy was caused predominantly by a superior specificity with the heart rate–monitoring application (96.7%, 95% CI 93.4%–96.7% v. 82.8%, 95% CI 77.1%–87.6%, $p < 0.001$).

Comparison of the heart rate–monitoring application and conventional plethysmography

To further evaluate its performance, we compared the overall diagnostic accuracy of the heart rate–monitoring application with that of conventional plethysmography. The application had a 94.0% accuracy compared to 93.5% with conventional plethysmography in all of the arteries that were assessed ($p = 0.7$). Although the heart rate–monitoring application showed superior accuracy relative to the modified Allen test, it was comparable to that of conventional plethysmography.

Adverse events

There were no procedural related complications during this study. Fourteen participants had an in-hospital major adverse cardiac event (i.e., myocardial infarction, stroke or death); 10 were in the modified Allen test cohort (5 patients with stroke, 1 with myocardial infarction and 4 deaths) and 4 were in the heart rate–monitoring application cohort (2 patients with myocardial infarctions and 2 deaths).

Interpretation

This proof of concept study implemented the photoplethysmographic capability widely available in smartphones for point-of-care evaluation of arterial patency — in this instance, for the assessment of dual circulation before radial artery access for cardiac catheterization or hemodynamic monitoring. We have shown that use of a heart rate–monitoring application has the potential to improve diagnostic accuracy relative to the modified Allen test — the widely used clinical standard. In our study, the incidence of ulnar artery occlusion as assessed by Doppler ultrasonography was low, and the increased diagnostic discriminatory power of the heart rate–monitoring application was primarily caused by a reduction in false-positive tests. Thus, our study

Table 3: Diagnostic accuracy of each test for ulnar and radial arteries

Parameter	Examined using the modified Allen test	Examined using the heart rate–monitoring application	*p* value†
Ulnar artery assessment, *n* = 736			
No. (%) of positive test results*	126 (17.1)	49 (6.7)	< 0.001
Overall diagnostic accuracy, % (95% CI)	84.0 (79.1 to 87.9)	92.1 (88.7 to 94.6)	0.002
Specificity, % (95% CI)	84.6 (79.6 to 88.5)	93.3 (90.2 to 95.5)	< 0.001
Sensitivity, % (95% CI)	40.0 (14.3 to 72.7)	37.5 (12.5 to 71.5)	0.9
Positive likelihood ratio (95% CI)	2.6 (–0.3 to 5.4)	5.6 (0.1 to 11.1)	
Negative likelihood ratio (95% CI)	0.7 (0.2 to 1.2)	0.7 (0.3 to 1.0)	
Radial artery assessment, *n* = 736			
No. (%) of positive test results*	102 (13.9)	30 (4.1)	< 0.001
Overall diagnostic accuracy, % (95% CI)	84.0 (76.6 to 87.6)	95.9 (93.4 to 97.5)	< 0.001
Specificity, % (95% CI)	85.6 (81.1 to 89.1)	96.9 (94.6 to 98.3)	< 0.001
Sensitivity, % (95% CI)	46.7 (25.4 to 69.2)	50.0 (23.1 to 76.9)	0.9
Positive likelihood ratio (95% CI)	3.2 (1.3 to 5.2)	16.4 (1.5 to 31.2)	
Negative likelihood ratio (95% CI)	0.6 (0.3 to 0.9)	0.5 (0.2 to 0.9)	
All arteries assessed, *n* = 1472			
No. (%) of positive test results*	228 (15.5)	79 (5.4)	< 0.001
Overall diagnostic accuracy, % (95% CI)	84.0 (79.9 to 87.4)	94.0 (91.9 to 95.6)	< 0.001
Specificity, % (95% CI)	85.1 (80.9 to 88.5)	95.1 (93.2 to 96.6)	< 0.001
Sensitivity, % (95% CI)	45.0 (26.4 to 65.2)	43.8 (23.9 to 65.9)	0.9
Positive likelihood ratio (95% CI)	3.0 (1.5 to 4.6)	9.0 (3.2 to 17.8)	
Negative likelihood ratio (95% CI)	0.6 (0.4 to 0.9)	0.6 (0.3 to 0.8)	

Note: CI = confidence interval.
*A positive test result indicates that there is inadequate collateral blood flow; this includes all true-positive and false-positive results.
†Values of *p* were generated using a generalized estimating equation model for the randomization arm parameter (i.e., modified Allen test versus heart rate–monitoring application) with binomial distribution using a logit link where the outcome was a correct test result.

shows the utility of a smartphone for point-of-care diagnostics with numerous clinical applications.

Mobile digital technology has been ubiquitously and rapidly integrated into workplaces, yet the development, evaluation and validation of smartphones for point-of-care diagnostics has lagged.[23] Barriers to the acceptance of mobile health technologies among health care providers include concerns regarding their efficacy and safety, ease of use, risk to patient confidentiality and diagnostic accuracy.[24] For example, a recent study highlighted inaccuracies in assessments of blood pressure by an application sold to nearly 150 000 users, by providing falsely reassuring blood pressure levels with hypertensive readings.[25] Although such cases warrant concern, considerable effort and resources are being invested in integrating diagnostics into smartphones for a wide array of conditions (e.g., Apple's ResearchKit).[26] The current report highlights that a smartphone application can outperform the current standard of care and provide incremental diagnostic yield in clinical practice. However, as with all new interventions and technologies, rigorous and systematic evaluation is needed before clinical integration.

Although not our primary goal, our study adds to the growing body of evidence that assessment of dual circulation by current standards is inadequate and may be irrelevant to the selection of patients for transradial access for cardiac catheterization. First, our findings show the low incidence of ulnar artery occlusion and the lack of sensitivity associated with abnormal results from the modified Allen test or heart rate–monitoring application. Thus, routine assessment will both inappropriately exclude a higher number of patients from the benefits of transradial access while simultaneously failing to identify a substantial proportion of those patients with inadequate ulnar reserve. Second, some studies have shown the presence of recruitable vascular reserve in the hand that can prevent clinically evident ischemia after mechanical manipulation of the radial artery.[27,28] This concept is supported clearly by studies that show the safety of using the homolateral ulnar artery for access in patients with occluded or unavailable radial artery access.[29,30] Finally, no substantial numbers of ischemic complications were reported in our study or in other RCTs that evaluated transradial access.[2,12,31] Thus, although the practice of assessment of dual circulation is prevalent and rooted in the

culture of medicine and guidelines, the practice remains without a reasonable foundation of evidence to support its use.

Performing the modified Allen test before radial artery access is recommended currently by numerous medical societies — including before transradial cardiac catheterization,[13,14] acquisition of arterial blood gases[15] and harvesting of the radial artery for coronary artery bypass grafting.[32] In addition, the reverse modified Allen test is performed commonly to assess for radial artery occlusion after transradial artery catheterization. Although Doppler ultrasonography remains the gold standard, the modified Allen test remains the predominant form of ulnar patency assessment because this test can be performed at point of care. Based on our results, if the modified Allen test is to be used, replacing it with a photoplethysmography-based assessment using a smartphone application has the potential to increase the number of patients eligible for radial artery access by 10% over standard clinical care.

Limitations

Our study has some limitations. As a proof of concept study, we wanted to show the utility of a smartphone to improve diagnostic accuracy, but it was not designed to detect any difference in ischemic adverse events from transradial access. However, given the low prevalence of clinically meaningful outcomes in transradial studies, adequately powered studies would be prohibitively large. Instead, if dual circulation assessment is to be used, our data support the use of the heart rate–monitoring application to achieve a higher diagnostic accuracy and specificity, thereby allowing clinicians to offer transradial access to a greater number of patients. We also used the absence of documented Doppler flow in the ulnar as the gold standard for inadequate ulnar collateral supply. In addition to static testing, methods that employ dynamic assessment of Doppler signals during radial artery compression have been described;[22] however, none of these techniques are associated with better prognostication of adequate collateral flow, and thus we elected to use the most extreme case (i.e., absence of flow) as our standard. Furthermore, all testing was performed by the same co-investigator, which predisposed our study to verification bias; however, randomly assigning participants to either the heart rate–monitoring application or the modified Allen test for the initial assessment and then comparing the result to our reference standard was important in mitigating this bias. Finally, all testing was performed using an iPhone 4s smartphone and a specific version of the heart rate–monitoring application. Given the differences in software and hardware specifications, these results are not necessarily generalizable to all smartphone platforms nor to different photoplethysmographic applications.

Conclusion

We found that using a smartphone heart rate–monitoring application to assess ulnar artery patency had superior diagnostic accuracy than the clinical standard (modified Allen test). Although this application is not certified at present for use in health care by any regulatory body, our study highlights the potential for smartphone-based diagnostics to aid in clinical decision-making at the patient's bedside.

References

1. Jin C, Li W, Qiao SB, et al. Costs and benefits associated with transradial versus transfemoral percutaneous coronary intervention in China. *J Am Heart Assoc* 2016;5:e002684.
2. Valgimigli M, Gagnor A, Calabró P, et al.; MATRIX Investigators. Radial versus femoral access in patients with acute coronary syndromes undergoing invasive management: a randomised multicentre trial. *Lancet* 2015;385:2465-76.
3. Andò G, Capodanno D. Radial access reduces mortality in patients with acute coronary syndromes: results from an updated trial sequential analysis of randomized trials. *JACC Cardiovasc Interv* 2016;9:660-70.
4. Ruzsa Z, Nemes B, Pintér L, et al. A randomised comparison of transradial and transfemoral approach for carotid artery stenting: RADCAR (RADial access for CARotid artery stenting) study. *EuroIntervention* 2014;10:381-91.
5. Staniloae CS, Mody KP, Sanghvi K, et al. Histopathologic changes of the radial artery wall secondary to transradial catheterization. *Vasc Health Risk Manag* 2009;5:527-32.
6. Yonetsu T, Kakuta T, Lee T, et al. Assessment of acute injuries and chronic intimal thickening of the radial artery after transradial coronary intervention by optical coherence tomography. *Eur Heart J* 2010;31:1608-15.
7. Uhlemann M, Mobius-Winkler S, Mende M, et al. The Leipzig prospective vascular ultrasound registry in radial artery catheterization: impact of sheath size on vascular complications. *JACC Cardiovasc Interv* 2012;5:36-43.
8. Rhyne D, Mann T. Hand ischemia resulting from a transradial intervention: successful management with radial artery angioplasty. *Catheter Cardiovasc Interv* 2010;76:383-6.
9. Ruzsa Z, Pinter L, Kolvenbach R. Anterograde recanalisation of the radial artery followed by transradial angioplasty. *Cardiovasc Revasc Med* 2010;11:266.e1-4.
10. de Bucourt M, Teichgraber U. Digital ischemia and consecutive amputation after emergency transradial cardiac catheter examination. *Cardiovasc Intervent Radiol* 2012;35:1242-4.
11. Bertrand OF, Rao SV, Pancholy S, et al. Transradial approach for coronary angiography and interventions: results of the first international transradial practice survey. *JACC Cardiovasc Interv* 2010;3:1022-31.
12. Valgimigli M, Campo G, Penzo C, et al. Transradial coronary catheterization and intervention across the whole spectrum of Allen test results. *J Am Coll Cardiol* 2014;63:1833-41.
13. Hamon M, Pristipino C, Di Mario C, et al.; European Association of Percutaneous Cardiovascular Interventions; Working Group on Acute Cardiac Care of the European Society of Cardiology; Working Group on Thrombosis on the European Society of Cardiology. Consensus document on the radial approach in percutaneous cardiovascular interventions: position paper by the European Association of Percutaneous Cardiovascular Interventions and Working Groups on Acute Cardiac Care** and Thrombosis of the European Society of Cardiology. *EuroIntervention* 2013;8:1242-51.
14. Caputo RP, Tremmel JA, Rao S, et al. Transradial arterial access for coronary and peripheral procedures: executive summary by the Transradial Committee of the SCAI. *Catheter Cardiovasc Interv* 2011;78:823-39.
15. *WHO guidelines on drawing blood: best practices in phlebotomy — Annex I: Modified Allen test*. Geneva: World Health Organization; 2010. Available: www.ncbi.nlm.nih.gov/books/NBK138652/ (accessed 2016 June 2).
16. Jarvis MA, Jarvis CL, Jones PRM, et al. Reliability of Allen's test in selection of patients for radial artery harvest. *Ann Thorac Surg* 2000;70:1362-5.
17. McGregor AD. The Allen test — an investigation of its accuracy by fluorescein angiography. *J Hand Surg Br* 1987;12:82-5.
18. Bertrand OF, Carey PC, Gilchrist IC. Allen or no Allen: that is the question! *J Am Coll Cardiol* 2014;63:1842-4.
19. Benit E, Vranckx P, Jaspers L, et al. Frequency of a positive modified Allen's test in 1,000 consecutive patients undergoing cardiac catheterization. *Cathet Cardiovasc Diagn* 1996;38:352-4.
20. Dorlas JC, Nijboer JA. Photo-electric plethysmography as a monitoring device in anaesthesia. Application and interpretation. *Br J Anaesth* 1985;57:524-30.
21. Barbeau GR, Arsenault F, Dugas L, et al. Evaluation of the ulnopalmar arterial arches with pulse oximetry and plethysmography: comparison with the Allen's test in 1010 patients. *Am Heart J* 2004;147:489-93.
22. Habib J, Baetz L, Satiani B. Assessment of collateral circulation to the hand prior to radial artery harvest. *Vasc Med* 2012;17:352-61.

23. Topol EJ, Steinhubl SR, Torkamani A. Digital medical tools and sensors. *JAMA* 2015;313:353-4.
24. Gagnon MP, Ngangue P, Payne-Gagnon J, et al. m-Health adoption by healthcare professionals: a systematic review. *J Am Med Inform Assoc* 2016;23:212-20.
25. Plante TB, Urrea B, MacFarlane ZT, et al. Validation of the instant blood pressure smartphone app. *JAMA Intern Med* 2016;176:700-2.
26. ResearchKit Studies. Baltimore: John Hopkins Medicine; 2016 Jan. 1 [updated]. Available: www.hopkinsmedicine.org/news/publications/insight/features/researchkit-studies (accessed 2016 Nov. 23).
27. Kim SY, Lee JS, Kim WO, et al. Evaluation of radial and ulnar blood flow after radial artery cannulation with 20- and 22-gauge cannulae using duplex Doppler ultrasound. *Anaesthesia* 2012;67:1138-45.
28. Ikizler M, Entok E, Ozdemir C, et al. Neurological status and tissue perfusion changes after radial artery harvesting for myocardial revascularization: importance of the Allen Test. *Thorac Cardiovasc Surg* 2007;55:99-103.
29. Kedev S, Zafirovska B, Dharma S, et al. Safety and feasibility of transulnar catheterization when ipsilateral radial access is not available. *Catheter Cardiovasc Interv* 2014;83:E51-60.
30. Kwan TW, Ratdiffe JA, Chaudhry M, et al. Transulnar catheterization in patients with ipsilateral radial artery occlusion. *Catheter Cardiovasc Interv* 2013;82:E849-55.
31. Mehta SR, Jolly SS, Cairns J, et al. Effects of radial versus femoral artery access in patients with acute coronary syndromes with or without ST-segment elevation. *J Am Coll Cardiol* 2012;60:2490-9.
32. Baikoussis NG, Papakonstantinou NA, Apostolakis E. Radial artery as graft for coronary artery bypass surgery: advantages and disadvantages for its usage focused on structural and biological characteristics. *J Cardiol* 2014;63:321-8.

Please see the following videos online:

Demonstration of the procedure for the modified Allen test

Procedure for photoplethysmography readings obtained using a heart rate–monitoring application and a smartphone

www.cmaj.ca/lookup/suppl/doi:10.1503/cmaj.170432/-/DC2

Competing interests: Roxana Mehran has received research grants paid to her institution from Astra Zeneca, The Medicines Company, Bristol-Myers Squibb/Sanofi, Eli Lilly and Company and Daiichi Sankyo outside the submitted work. She has received consultant fees from AstraZeneca, Bayer, CSL Behring, Janssen Pharmaceuticals, Merck & Co., The Medicines Company and WaterMark Consulting. She has received renumeration as a member of the advisory boards of Abbott Laboratories, AstraZeneca, Boston Scientifc, Covidien, Janssen Pharmaceuticals, Merck & Co., The Medicines Company and Sanofi-Aventis. She holds less than 1% equity in Claret Medical and Elixir Medical Corporation. No other competing interests were declared.

This article has been peer reviewed.

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Contributors: All of the authors contributed substantially to the conception or design of the work, drafted the work and revised it critically for important intellectual content, gave final approval of the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Data sharing: The data used for this analysis are available upon request to Benjamin Hibbert with details of the proposed analysis. A request will be reviewed and authorized once it is deemed scientifically justified.

Disclaimer: George Wells is a biostatistical consultant for *CMAJ* and was not involved in the editorial decision-making process for this article.

Accepted: Dec. 6, 2017

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Appendix Q

Sex bias is increasingly prevalent in preclinical cardiovascular research: implications for translational medicine and health equity for women

Citation information:

Ramirez FD, Motazedian P, **Jung RG**, Di Santo P, MacDonald Z, Simard T, Hibbert B. Sex bias is Increasingly Prevalent in Preclinical Cardiovascular Research: Implications for Translational Medicine and Health Equity for Women. **(2017)**. *Circulation. 135(6):625-626.*

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Sex Bias Is Increasingly Prevalent in Preclinical Cardiovascular Research: Implications for Translational Medicine and Health Equity for Women

Wolters Kluwer

Author: Benjamin Hibbert, George A. Wells, Vivian Welch, et al

Publication: Circulation

Publisher: Wolters Kluwer Health, Inc.

Date: Feb 7, 2017

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RESEARCH LETTER

Sex Bias Is Increasingly Prevalent in Preclinical Cardiovascular Research: Implications for Translational Medicine and Health Equity for Women

A Systematic Assessment of Leading Cardiovascular Journals Over a 10-Year Period

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Ensuring that women are adequately represented in clinical trials is recognized as essential for sex equity in health. However, the use of female animal models and sex-based reporting have not been equally enforced in preclinical stages of research, which often precede and inform clinical trials. In 2014, the National Institutes of Health announced that it would require that sex be considered as a biological variable in applications for preclinical research funding,[1] yet a reluctance to include female animal models in preclinical experiments persists. Inappropriately inferring experimental findings to both sexes when a single sex is studied or when sex is not specified has the potential to disadvantage women by skewing our understanding of disease processes toward male-predominant patterns and by reducing the likelihood of female-specific therapeutics advancing to the clinical realm. We therefore systematically examined all preclinical cardiovascular studies published in American Heart Association journals with archives spanning at least 10 years (*Circulation*, *Circulation Research*, *Hypertension*, *Stroke*, and *Arteriosclerosis, Thrombosis, and Vascular Biology* [*ATVB*]) for evidence of sex bias.

Full articles published between July 2006 and June 2016 and reporting original data from in vivo experiments in nonhuman mammals on pathophysiology, genetics, or therapeutic interventions directly relevant to a specific cardiovascular disorder in humans were included. Studies on physiological or genetic characteristics were included if they proposed potential therapeutic applications or implications of the study findings. Each journal article was independently reviewed and prespecified data were extracted by using standardized case report forms by 2 authors, including the cardiovascular disease investigated, animal model(s) used and their sex, whether study samples were sex matched (in studies using both sexes), whether at least 1 study result was reported by sex, and whether the use of a single sex was reported as a limitation (in single-sex studies). Interrater agreement was calculated using the Cohen κ-statistic and percentage agreement. Discrepancies were resolved by consensus or independent adjudication. Categorical variables were compared via χ^2 tests. Temporal patterns were evaluated via Pearson correlation or Cochrane-Armitage trend tests. All analyses were performed by using SAS 9.4 (SAS Institute Inc) with the use of an α-level of 0.05 to define statistical significance.

Of 28636 articles screened, 3396 met inclusion criteria and were analyzed. Interrater agreement for study inclusion before resolution was 94.5% (κ=0.72; 95% confidence interval, 0.70–0.73). The sex of the animals used was not reported in 20.0% of studies. Males were exclusively used in 71.6% of studies in which sex was reported, whereas females were exclusively used in 12.9% and both sexes in 15.5%. Sex matching of animals was reported in 17.1% of studies that included both sexes. Restricting this analysis to the 988 studies of therapeutic interventions did not appreciably change these distributions. When stratified by the cardiovascular disease studied, males were exclusively used significantly more often than females or both sexes in all cases, with the exception of atherosclerosis and arrhythmia. When stratified by the animal model

*Dr Ramirez and P. Motazedian contributed equally.

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Key Words: bias (epidemiology) ■ cardiovascular diseases ■ publications ■ research ■ sex

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CORRESPONDENCE

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used, males were exclusively used significantly more often when mice, rats, rabbits, or a combination of animal models was used (accounting for 95.0% of studies).

The number of preclinical studies published yearly over the past decade has remained stable; however, there has been a significant increase in the proportion using exclusively males and a significant decrease in the proportion using exclusively females. There have not been significant changes in the proportions of studies using both sexes or reporting the sex of the animals used (Figure). These trends were unchanged when studies in which sex was not specified were excluded.

Sex-based reporting of results occurred in 35.1% of studies in which sex was reported using the liberal criterion of any results being reported in reference to the sex of the animals used at least once. There was no appreciable difference in the prevalence of sex-based reporting before and after the publication of planned National Institutes of Health policies to improve sex inclusion in preclinical research[1] (34.9% versus 35.7%, respectively). Of the 2297 studies in which only 1 sex was reportedly used, 1.6% mentioned this aspect of the study design as a potential limitation.

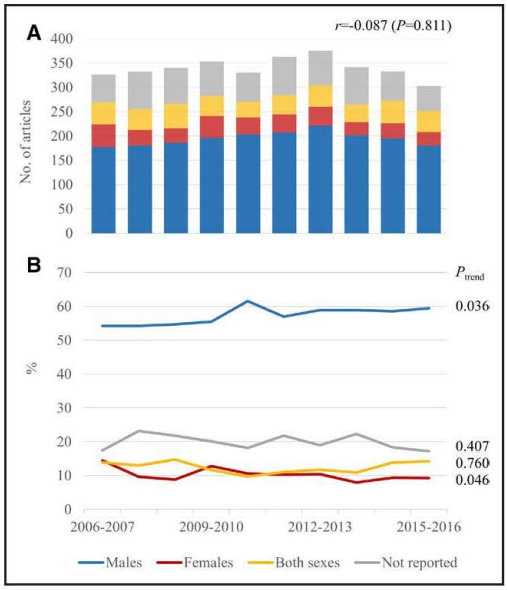


Figure. Temporal patterns in the sex of animals used in preclinical cardiovascular research over a 10-year period.

A, Absolute number of preclinical studies published and sex of animals used as a function of time. *r* denotes Pearson correlation coefficient for the total number of studies over time. **B,** Sex of animals used in preclinical studies over time (%). P_{trend} calculated for the proportion of studies using exclusively males, exclusively females, both sexes, and not reporting the sex of the animals used over time.

Although the need to include women in clinical trials is now a well-established requirement to enhance external validity, analogous standards have not gained a foothold in preclinical stages. Reasons put forward for the preferential use of males include increased variability attributable to fluctuating gonadal hormone levels throughout the estrous cycle and sample size implications of planning sex-based analyses.[2,3] Evidence to support these concerns is limited for most experimental settings, however.[3–5]

Our review of preclinical research published in leading cardiovascular journals over the past 10 years demonstrates that sex bias is prevalent and increasing, contrasting with advances made in clinical research designs and reporting. Concerted efforts on the part of researchers, journal editors, peer reviewers, universities, industry, professional organizations, and patient advocacy groups are urgently needed to address this discrepancy.

DISCLOSURES

None.

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FOOTNOTES

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REFERENCES

1. Clayton JA, Collins FS. Policy: NIH to balance sex in cell and animal studies. *Nature*. 2014;509:282–283.
2. Zucker I, Beery AK. Males still dominate animal studies. *Nature*. 2010;465:690. doi: 10.1038/465690a.
3. Clayton JA. Studying both sexes: a guiding principle for biomedicine. *FASEB J*. 2016;30:519–524. doi: 10.1096/fj.15-279554.
4. Prendergast BJ, Onishi KG, Zucker I. Female mice liberated for inclusion in neuroscience and biomedical research. *Neurosci Biobehav Rev*. 2014;40:1–5. doi: 10.1016/j.neubiorev.2014.01.001.
5. Itoh Y, Arnold AP. Are females more variable than males in gene expression? Meta-analysis of microarray datasets. *Biol Sex Differ*. 2015;6:18. doi: 10.1186/s13293-015-0036-8.

Circulation. 2017;135:625–626. DOI: 10.1161/CIRCULATIONAHA.116.026668

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Appendix R

Evaluation of cobalt and chromium levels following implantation of cobalt chromium coronary stents: a pilot study

Citation information:

Di Santo P, Motazedian P, **Jung RG**, Simard T, Ramirez FD, Chong AY, Glover C, Hibbert B, Dwivedi G. Evaluation of cobalt and chromium levels following implantation of coronary stents: a pilot study. **(2017).** *Heart, Lung, Circulation Journal.*

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Evaluation of Cobalt and Chromium Levels Following Implantation of Cobalt Chromium Coronary Stents: A Pilot Study

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Publication: Heart, Lung and Circulation

Publisher: Elsevier

Date: June 2018

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Heart, Lung and Circulation (2018) **27**, 763–766
1443-9506/04/$36.00
https://doi.org/10.1016/j.hlc.2017.12.002

BRIEF COMMUNICATION

Evaluation of Cobalt and Chromium Levels Following Implantation of Cobalt Chromium Coronary Stents: A Pilot Study

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Received 27 May 2017; accepted 3 December 2017; online published-ahead-of-print 14 December 2017

Background Large increases in myocardial trace elements may adversely affect metabolism and become detrimental to cardiac function. Percutaneous coronary intervention (PCI) allows for the revascularisation of obstructive coronary artery disease using drug-eluting stents. These stents are comprised of a metallic stent backbone covered in an engineered polymer which delivers a drug over a prescribed period to the vessel wall. Given the potential implications of trace metal accumulation within the myocardium, our goal is to determine if metallic coronary stents are able to cause detectable elevations in serum cobalt and/or chromium levels.

Methods This study was a single centre, observational, pilot study with 20 patients who underwent planned PCI with implantation of a cobalt chromium drug eluting stent. Serum blood samples were drawn at baseline prior to PCI, 4 hours post-stent deployment and at the time of routine follow-up after PCI. All blood samples were analysed for cobalt and chromium concentrations. The primary outcome of this study was the difference in serum cobalt and chromium levels at routine clinical follow-up.

Results The mean follow up was 64.1 $\pm$ 17.3 days. There was no difference in serum cobalt levels when comparing baseline and routine clinical follow up (3.32 $\pm$ 2.14 nmol/L vs. 3.14 $\pm$ 1.00 nmol/L, $p = 0.99$) nor in chromium levels (4.24 $\pm$ 2.31 nmol/L vs. 2.82 $\pm$ 1.22 nmol/L, $p = 0.11$). There was also no difference between baseline and 4 hours post-PCI serum concentrations.

Conclusions Percutaneous coronary intervention with cobalt chromium coronary stents does not appear to cause an elevation in these trace element serum concentrations.

Keywords Cobalt • Chromium • Cardiomyopathy • Trace metals • Percutaneous coronary intervention • Drug eluting stents

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Introduction

Trace elements play crucial roles in myocardial metabolism and disruptions in the levels of these elements may lead to cardiac dysfunction. In particular, myocardial cobalt accumulation is known to be associated with dilated cardiomyopathy [1]. First described in 1967 [2], cobalt cardiomyopathy has been increasingly studied [3], including elevated cobalt levels stemming from metal-on-metal hip arthroplasties leading to cardiologic, neurologic, and endocrinologic dysfunction [4]. Thresholds of what constitutes organ specific toxic concentrations of cobalt have not yet been well defined [5] although levels above seven parts per billion, which is equivalent to 119 nmol/L cobalt or 134.5 nmol/L chromium, are considered to be unsafe [6].

Percutaneous coronary intervention (PCI) remains a standard of care for the revascularisation of obstructive coronary artery disease. The current state of the art in coronary stenting involves modern drug-eluting stents—comprised of a metallic stent backbone covered in an engineered polymer which delivers drugs over a prescribed period to the vessel wall [7]. Advancements in the metallic struts as the backbone for treating obstructive coronary artery disease include the use of stainless steel, platinum chromium, and cobalt chromium metals. In particular, cobalt chromium stents are commonly used within many centres worldwide.

Given the potential implications of trace metal accumulation within the myocardium, our goal is to determine if coronary stents, which are only micrometres in thickness and millimetres in length, are able to cause detectable elevations in cobalt and/or chromium serum levels.

Methods

Design

This study was a single centre, observational, pilot study with 20 patients who were undergoing planned PCI with implantation of a cobalt-chromium drug eluting stent, either with Xience V (Abbott Vascular, Santa Clara, California, USA) or Resolute Integrity (Medtronic, Santa Rosa, California, USA) stents. Informed consent was obtained from each patient. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the institution's human research committee.

Blood Samples

Blood samples were drawn at baseline prior to PCI, 4 hours post-stent deployment and at the time of routine follow-up after PCI. The blood samples were spun at 3000 rpm for 10 minutes and then stored in a −20° C freezer until sampling was performed. All samples were analysed for quantification of serum cobalt and chromium levels in a single third party laboratory.

Inclusion/Exclusion Criteria

The inclusion criteria for this study were broad and included all patients greater than 18 years of age awaiting PCI with cobalt-chromium stents. Patients were excluded if they had previous PCI, non-cobalt chromium stents deployed at time of their procedure, previous cobalt-based arthroplasties,

Table 1 Patient and procedural characteristics.

	Participants
Men	14 (70%)
Age, y	62.9 (8.8)
Height, m	1.7 (0.1)
Weight, kg	88.7 (16.0)
Body mass index, kg/m^2	29.4 (4.4)
Medical co-morbidities/history	
Hypertension	13 (65%)
Diabetes	2 (10%)
Smoking	8 (40%)
Family history of premature CAD*	3 (15%)
Dyslipidaemia	11 (55%)
Previous myocardial infarction	4 (20%)
Previous CABG	1 (5%)
Previous stroke	1 (5%)
Chronic kidney disease	0 (0%)
Dialysis	0 (0%)
Congestive heart failure	1 (5%)
Medications	
ACEI/ARB	8 (40%)
Beta blocker	10 (50%)
Statin	15 (75%)
ASA	18 (90%)
Clopidogrel/Ticagrelor	17 (85%)
Therapeutic anticoagulation	3 (15%)
Nitrate	3 (15%)
Indication for PCI	
Stable angina	10 (50%)
Acute coronary syndrome	7 (35%)
Chronic total occlusion	3 (15%)
Location	
Right coronary artery	8 (40%)
Left anterior descending	7 (35%)
Left circumflex	5 (25%)
Total no. stents inserted	1.95 (0.8)
Total length of stents, mm	56.7 (23.6)
Deployment pressure, atm	15.0 (2.8)
Maximum post-dilatation pressure, atm	18.9 (3.9)

Abbreviations: ACEI/ARB, angiotensin converting enzyme inhibitor/angiotensin receptor blocker; CABG, coronary artery bypass surgery; CAD, coronary artery disease; PCI, percutaneous coronary intervention.
*Defined as having a first-degree relative with clinically overt CAD (myocardial infarction, angina pectoris, need for coronary artery revascularisation) before age 55 (men) or 65 (women).

hard metal workers, or unwillingness to provide informed consent.

Outcomes

The primary outcome of this study was the difference in serum cobalt and chromium levels at routine clinical follow-up. The secondary outcome of this study was the difference in serum trace metal levels at 4 hours post-PCI.

Statistical Analysis

As this was a pilot study, a sample size calculation was not performed. All continuous variables are reported as mean ± SD. Categorical variables are described as number (%). Analyses were performed using a paired t-test for both primary and secondary outcomes. All analyses were performed using SPSS version 24.0 (IBM Corporation, Armonk, New York, USA).

Results

Baseline clinical characteristics were reflective of a general cardiology population (Table 1). The indication for PCI was stable angina in the majority of cases, with only 25% acute coronary syndromes and 15% for chronic total occlusions. The mean stent length was 56.7 ± 23.6 mm with 1.95 ± 0.8 stents deployed per patient. Stent deployment pressure was 14.8 ± 2.8 atm with post-stent deployment dilatation pressures averaging 19.3 ± 3.8 atm.

The mean follow-up in this study was 64.1 ± 17.3 days. There was no difference in serum cobalt levels when comparing baseline and routine clinical follow up (3.32 ± 2.14 nmol/L vs. 3.14 ± 1.00 nmol/L, $p = 0.99$) nor in chromium levels (4.24 ± 2.31 nmol/L vs. 2.82 ± 1.22 nmol/L, $p = 0.11$, Figure 1). There was also no difference in cobalt levels when comparing baseline and 4 hours post-PCI (3.32 vs. 3.21 nmol/L, $p = 0.55$) and chromium levels (4.24 vs. 2.82, $p = 0.11$), respectively.

Discussion

Trace element accumulation within the myocardium can be detrimental to overall cardiac physiology and function. Certainly, there has been considerable interest into the accumulation of cobalt within myocardium which can lead to a distinctive, rapidly progressive but reversible depression in cardiac function [3]. In this pilot study, we demonstrate that PCI with cobalt-chromium coronary stents does not lead to an increase in the serum concentrations of cobalt or chromium metals.

Recently, there has been a resurgence in interest in cobalt cardiomyopathy. Several sources of cobalt have been recognised in the past including cobalt-chloride tablets for the treatment of anaemia, occupational exposures, beer, and metal alloys for health care usage [3]. Specifically, there has been an increased recognition of adverse effects of metal-on-metal total hip arthroplasties which have led to recommendations from several regulatory bodies, including in the United Kingdom [6], the US Food & Drug Administration [8], Health Canada [9], and the Therapeutic Goods Administration of Australia [10], to monitor patients closely for evidence of potential local and systemic toxicities. As a result of these concerns, the rate of metal-on-metal total hip arthroplasties has been steadily decreasing [4].

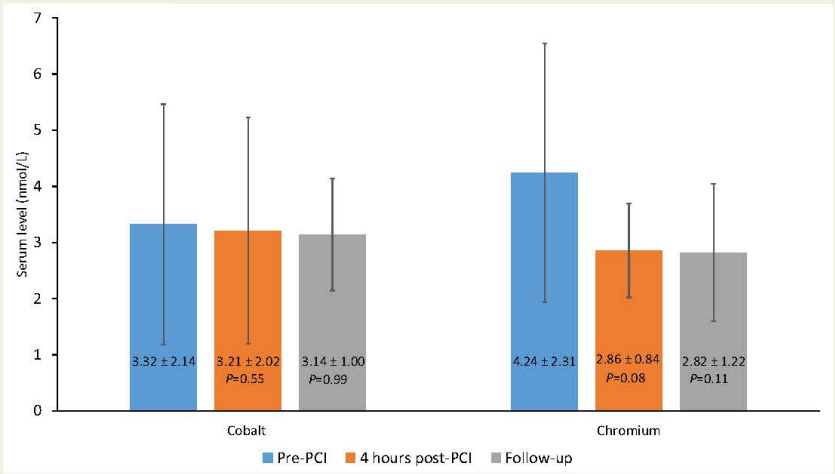


Figure 1 Serum cobalt and chromium levels at baseline, 4-hours post-PCI, and at routine clinical follow up showing no difference following implantation of cobalt-chromium stents. p Values are for comparison to baseline levels.

As a pilot study, our sample size was insufficiently powered to detect clinical outcomes such as development of dilated cardiomyopathy. Nonetheless, the levels of cobalt (3.14 ± 1.00 nmol/L) and chromium (2.82 ± 1.22 nmol/L) at routine follow-up are well below the unsafe level of seven parts per billion, which is equivalent to 119 nmol/L cobalt or 134.5 nmol/L chromium. Accordingly, further studies investigating coronary stents as a potential cardiotoxic source of trace metals are not warranted.

Conclusions

Percutaneous coronary intervention with cobalt chromium coronary stents does not appear to cause an elevation in these trace element serum concentrations.

Declaration

The authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

Conflicts of Interest

None.

Acknowledgement

This study was funded by the Patient Quality and Safety Committee Fund at the University of Ottawa Heart Institute.

References

[1] Frustaci A, Magnavita N, Chimenti C, Caldarulo M, Sabbioni E, Pietra R, et al. Marked elevation of myocardial trace elements in idiopathic dilated cardiomyopathy compared with secondary cardiac dysfunction. J Am Coll Cardiol 1999;33(6):1578–83.
[2] Morin Y, Daniel P. Quebec beer-drinkers' cardiomyopathy: etiological considerations. CMAJ 1967;97(15):926–8.
[3] Packer M. Cobalt cardiomyopathy. Circ Heart Fail 2016;9(12).
[4] Cheung AC, Banerjee S, Cherian JJ, Wong F, Butany J, Gilbert C, et al. Systemic cobalt toxicity from total hip arthroplasties. Bone Joint J 2016;98-B(1):6–13.
[5] Zywiel MG, Cherian JJ, Banerjee S, Cheung AC, Wong F, Butany J, et al. Systemic cobalt toxicity from total hip arthroplasties. Bone Joint J 2016;98-B(1):14–20.
[6] No authors listed. Metal-on-metal (MoM) hip replacements – updated advice with patient follow ups. [Last accessed on 15 April 2017]; Available from: https://www.gov.uk/drug-device-alerts/medical-device-alert-metal-on-metal-mom-hip-replacements-updated-advice-with-patient-follow-ups.
[7] Simard T, Hibbert B, Ramirez FD, Froeschl M, Chen YX, O'Brien ER. The evolution of coronary stents: a brief review. Can J Cardiol 2014;30(1):35–45.
[8] No authors listed. Information for all health care professionals who provide treatment to patients with a metal-on-metal hip implant. [Last accessed on 15 April 2017]; Available from: https://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/ImplantsandProsthetics/MetalonMetalHipImplants/ucm241744.htm.
[9] No authors listed. Metal-on-metal hip implants – information for orthopaedic surgeons regarding patient management following surgery – for health professionals. [Last accessed on 15 April 2017]; Available from: http://www.healthycanadians.gc.ca/recall-alert-rappel-avis/hc-sc/2012/15835a-eng.php.
[10] No authors listed. Metal-on-metal hip replacement implants. [Last accessed on 15 April 2017]; Available from: https://www.tga.gov.au/metal-metal-hip-replacement-implants.

Appendix S

Methodological rigor in preclinical cardiovascular studies

Citation information:

Ramirez FD, Motazedian P, **Jung RG**, Di Santo P, MacDonald ZD, Moreland R, Simard T, Clancy AA, Russo JJ, Welch VA, Wells GA, Hibbert B. Methodological Rigor in Preclinical Cardiovascular Studies: Targets to Enhance Reproducibility and Promote Research Translation. **(2017)**. *Circulation Research*. 120(12):1916-1926.

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Methodological Rigor in Preclinical Cardiovascular Studies

Author: F. Daniel Ramirez, Pouya Motazedian, Richard G. Jung, et al

Publication: Circulation Research

Publisher: Wolters Kluwer Health, Inc.

Date: Apr 3, 2017

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Methodological Rigor in Preclinical Cardiovascular Studies

Targets to Enhance Reproducibility and Promote Research Translation

F. Daniel Ramirez, Pouya Motazedian, Richard G. Jung, Pietro Di Santo, Zachary D. MacDonald, Robert Moreland, Trevor Simard, Aisling A. Clancy, Juan J. Russo, Vivian A. Welch, George A. Wells, Benjamin Hibbert

Rationale: **Methodological sources of bias and suboptimal reporting contribute to irreproducibility in preclinical science and may negatively affect research translation. Randomization, blinding, sample size estimation, and considering sex as a biological variable are deemed crucial study design elements to maximize the quality and predictive value of preclinical experiments.**

Objective: **To examine the prevalence and temporal patterns of recommended study design element implementation in preclinical cardiovascular research.**

Methods and Results: **All articles published over a 10-year period in 5 leading cardiovascular journals were reviewed. Reports of in vivo experiments in nonhuman mammals describing pathophysiology, genetics, or therapeutic interventions relevant to specific cardiovascular disorders were identified. Data on study design and animal model use were collected. Citations at 60 months were additionally examined as a surrogate measure of research impact in a prespecified subset of studies, stratified by individual and cumulative study design elements. Of 28 636 articles screened, 3396 met inclusion criteria. Randomization was reported in 21.8%, blinding in 32.7%, and sample size estimation in 2.3%. Temporal and disease-specific analyses show that the implementation of these study design elements has overall not appreciably increased over the past decade, except in preclinical stroke research, which has uniquely demonstrated significant improvements in methodological rigor. In a subset of 1681 preclinical studies, randomization, blinding, sample size estimation, and inclusion of both sexes were not associated with increased citations at 60 months.**

Conclusions: **Methodological shortcomings are prevalent in preclinical cardiovascular research, have not substantially improved over the past 10 years, and may be overlooked when basing subsequent studies. Resultant risks of bias and threats to study validity have the potential to hinder progress in cardiovascular medicine as preclinical research often precedes and informs clinical trials. Stroke research quality has uniquely improved in recent years, warranting a closer examination for interventions to model in other cardiovascular fields. (*Circ Res*. 2017;120:1916-1926. DOI: 10.1161/CIRCRESAHA.117.310628.)**

Key Words: animal models ■ bias ■ biomedical research ■ cardiovascular diseases ■ reproducibility of results

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Preclinical research and clinical therapy development exist in a symbiotic continuum yet are guided by different reporting standards, regulatory forces, and reward systems.[1,2] Consequent discrepancies in study designs and analytical methods can compromise scientific validity,[3] result in irreproducible results (particularly from preclinical experiments using animal models[2]), and may contribute to the high rate of attrition seen in early stages of clinical development.[4,5] Improved methodological rigor and transparent reporting practices have therefore been advocated to improve the predictive value of animal model data.[2,6–10]

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The issue of irreproducibility in preclinical research and its impact are well-recognized in academic circles[2,11–14] and

Original received January 12, 2017; revision received March 11, 2017; accepted March 31, 2017. In February 2016, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 15.4 days.

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The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.117.310628/-/DC1.

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Novelty and Significance

What Is Known?

- Preclinical research often precedes and informs clinical trials.
- Randomization, blinding, sample size estimation, and considering sex as a biological variable are considered crucial study design elements to maximize the predictive value of preclinical experiments.

What New Information Does This Article Contribute?

- Key study design elements are rarely implemented in preclinical cardiovascular research.
- The implementation of these elements has not appreciably improved over the past decade, except in stroke research.
- Methodological shortcomings in preclinical experiments may be overlooked when researchers design subsequent studies.

Preclinical and clinical research are guided by different methodological standards. Resultant discrepancies in study designs can compromise scientific validity, give rise to experimental irreproducibility, and may contribute to failures in research translation. Randomization, blinding, sample size estimation, and considering sex as a biological variable have been identified as key study design elements to promote rigor and reproducibility of animal experiments and research relevance to both men and women. However, there are limited data on the extent of suboptimal methodological rigor in preclinical research. We identified and analyzed 3396 preclinical studies in leading cardiovascular journals over a 10-year period, including citation counts at 60 months as a surrogate measure of research impact for papers published in the first 5 years. We show that these design elements were rarely implemented—a situation that has not substantially improved over the past decade, except in stroke research. The individual and cumulative implementation of these design elements was not associated with increased citations. Together, these data indicate that methodological shortcomings are prevalent in preclinical cardiovascular research and suggest that they may be overlooked when researchers design subsequent studies.

Nonstandard Abbreviations and Acronyms	
CVD	cardiovascular disorder
NIH	National Institutes of Health

increasingly so among the lay public[15–17] and the biopharmaceutical industry.[13,18] In 2011, Bayer HealthCare published an analysis of 67 in-house target identification and validation projects in the fields of oncology, women's health, and cardiovascular disease over a 4-year period, reporting that the published data were reproducible in less than one third of cases and that in nearly two thirds inconsistencies either prolonged the validation process or led to project termination.[11] In 2012, Amgen similarly published the results of their attempt to reproduce 53 "landmark" studies in the fields of hematology and oncology, succeeding in only 6 cases even after contacting the original authors for guidance, exchanging reagents, and in certain instances repeating experiments in the laboratory of the original investigators.[12] Some findings could not be reproduced even by the original investigators in their own laboratories when the experiments were repeated in a blinded fashion.[19] Disconcertingly, some of the irreproducible research were deemed to have prompted clinical studies. Others have likewise described candidate therapies that have advanced to testing in humans despite irreproducible or inconsistent results in animals.[8,20,21]

Since the mid-1990s, pharmaceutical research and development productivity has declined with decreasing numbers of medications approved, increasing attrition rates at all stages of research, and lengthening development times despite rising expenditures.[4] This decline has been attributed, in part, to flawed preclinical research methodology, highlighting the importance of preclinical research for advancing clinical care, but equally portending a waning confidence in animal model data to identify promising therapeutic targets.[5,7,11,12,22,23] Cardiovascular research and development, in particular, has fared poorly in recent years relative to other disease categories, exhibiting the greatest decline as a percentage of total projects[4] and among the lowest success rates and likelihood of approval at all phases of clinical trials.[24] Moreover, research and development investment patterns are increasingly deterring incremental innovation (for instance, improving upon available effective therapies). Instead, increasing focus is being placed on novel disease targets with higher revenue potential but at higher risk of failure (eg, specific cancers).[4] Poor reproducibility in preclinical research coupled with this increasingly unfavorable landscape for successful cardiovascular therapy development could, therefore, substantially hinder progress in cardiovascular care.

In response to the above systematic issues and in collaboration with editors from major journals, funding agencies, and scientific leaders, the National Institutes of Health (NIH) proposed a set of reporting guidelines and funding policies to improve the reproducibility and rigor of preclinical research.[25] A core set of standards first proposed by the NIH's National Institute of Neurological Disorders and Stroke were adopted, which identified randomization, blinding, sample size estimation, and data handling as minimum reporting requirements to promote transparency in animal studies.[7] In addition, the NIH announced that it would require that sex be considered as a biological variable in applications for preclinical research funding.[26] These principles and guidelines have been endorsed by prominent academic societies, associations, and journals, including all American Heart Association (AHA) journals,[25] with evidence of editorial commitment to complying.[27,28] However, there are limited data on the extent of suboptimal methodological rigor or incomplete reporting in preclinical cardiovascular science and therefore no baseline from which to gauge progress. A commitment to improving the quality and impact of preclinical research and to maintaining the trust of public and private stakeholders requires transparency on current and future states of scientific practice.[13] We, therefore, reviewed all preclinical cardiovascular studies published in leading AHA journals over the past decade to determine the prevalence of randomization, blinding, sample size estimation, and inclusion of both sexes and trends in these practices over time.

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Methods

As previously described,[29] all preclinical cardiovascular studies published in AHA journals with archives spanning at least 10 years were reviewed. Five journals met these criteria: *Circulation*; *Circulation Research*; *Hypertension*; *Stroke*; and *Arteriosclerosis, Thrombosis, and Vascular Biology* (*ATVB*). All reports published during a 10-year period (July 2006 to June 2016) were screened. Studies were included if they represented original research published as full manuscripts; reported results of in vivo experiments in nonhuman mammals; and described pathophysiology, genetics, or therapeutic interventions that were stated to be directly relevant to a specific cardiovascular disorder (CVD) in humans (see prespecified list of CVDs below). Studies on physiological and genetic characteristics were included if potential therapeutic applications or implications of the study findings were proposed in the article. These criteria are consistent with previously proposed definitions of preclinical ("confirmatory" or "proof-of-concept") experiments.[7,30,31] CVDs of interest included atherosclerosis or vascular homeostasis, arterial aneurysms or dissections, myocardial infarction, valvular disease, cardiomyopathy or heart failure, cardiac transplantation, pulmonary hypertension, cardiac arrhythmia, stroke, resuscitative medicine, hypertension, metabolic or endocrine diseases, and hematologic disorders (including thrombosis). Studies deemed to report on clinically relevant cardiovascular conditions but not falling into one of the prespecified categories could be included at the journal reviewers' discretion (category "other"). Each journal article was independently reviewed and data extracted using standardized case report forms by 2 reviewers, allowing for the assessments of inter-rater agreement. To permit our team to review a large volume of articles, we allowed for studies to be excluded as soon as it was clear that a manuscript violated any of our inclusion criteria, which was most often because of the model used (eg, zebrafish or humans) or because they were published in formats other than full manuscripts (eg, conference abstracts). The specific inclusion criterion/criteria that was/were deemed to have been violated for each excluded article, therefore, varied according to journal reviewer and were often multiple (eg, a nonmammalian animal model was used and no specific reference to therapeutic implications/applications was made). Discrepancies were resolved by consensus or by an independent adjudicator before building a final locked database for analysis.

Pre-specified data, including the date of publication, CVD investigated, animal model(s) used and their sex, whether animals were randomized to treatment groups, whether any blinding was implemented (concealed allocation or blinded outcome assessment), and whether a priori sample size/power estimations were performed were collected. Subgroup analyses restricted to studies of therapeutic interventions and by CVD studied were performed as were post hoc comparisons of these practices before and after the publication of NIH guidelines and policies for reporting preclinical research and the implementation of a "Basic Science checklist" by *Stroke*,[28] which is purported to have improved the quality and designs of preclinical studies published in that journal.[32]

Finally, the number of citations has been used as a surrogate measure of research impact and influence.[33–37] Therefore, Scopus (Elsevier) was queried to identify original research articles that cited preclinical cardiovascular studies published between July 2006 and June 2011. This 5-year period was selected as it ensured that each article had ≥5 years of citation data available in June 2016. Reviews, conference papers, editorials, letters, books/book chapters, and errata were excluded. Scopus was selected because it is the largest database available for citation analysis and retrieves a greater number of citations when compared with others.[38] Prespecified analyses of citations at 60 months after the index publication were performed, stratified by individual and cumulative study design elements and adjusted for journal of publication, year of publication, and CVD studied. Given the possibility that certain publications may have garnered attention in the short-term but could have ultimately been disproven or deemed irreproducible, a sensitivity analysis of citation counts at 36 months was also undertaken as this time point approximates contemporary mean time-to-retraction.[39]

Categorical variables are reported as number (%) and were compared via χ^2 tests. Continuous variables are reported as median (interquartile range) and were compared using Wilcoxon signed-rank or Kruskal–Wallis tests. Inter-rater agreement was calculated using Cohen κ statistic and percent agreement. Temporal patterns in the proportions of studies reporting randomization, blinding, and sample size estimations were evaluated via Cochrane–Armitage trend tests and journal-specific logistic regression models adjusting for CVD studied and animal model used when the number of events per predictor variable was adequate.[40–42] For the latter adjusted analyses, backward elimination was used for model building using a criterion of $P<0.20$ for specific CVD and animal model predictor variable inclusion. Associations between study design elements and citation counts were examined via stratification and multivariable linear regression. Non-normally distributed variables were log-transformed, when required. All analyses were performed using SAS 9.4 (SAS Institute Inc, Cary, NC) using a 2-tailed α level of 0.05 to define statistical significance.

Results

Study Selection and Characteristics

As previously described,[29] of 28636 articles screened, 3396 met inclusion criteria and were analyzed (Figure 1). Inter-rater

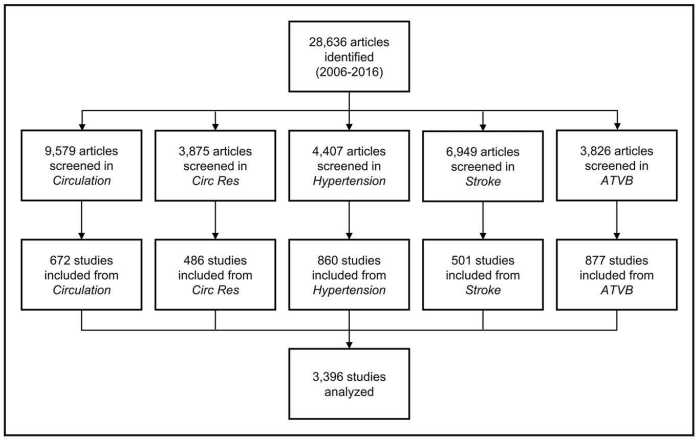


Figure 1. Literature search and results. *ATVB* indicates *Arteriosclerosis, Thrombosis and Vascular Biology*; and *Circ Res*, *Circulation Research*.

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agreement for study inclusion before resolution was 94.5% (κ=0.72; 95% CI, 0.70–0.73). Atherosclerosis, hypertension, stroke, and cardiomyopathy/heart failure were the most commonly studied CVDs (range 14.2%–19.5%). Most other CVDs were the focus of <5.0% of studies. Ten studies on resuscitative medicine were identified and were included in the "other" category, which comprised 3.0% of studies. Nearly one third of studies examined a therapeutic intervention. Mice or rats were most often used by researchers (used in 89.8% of studies) whereas guinea pigs, gerbils, or hamsters were used in <0.2%. Multiple animal models were used in 2.8% of studies (Table).

Randomization

Randomization of animals was reported in 740 studies (21.8%) overall, but was more frequently noted in the subset examining therapeutic interventions (38.3% versus 15.0%, $P<0.0001$). When studies were stratified by CVD studied, significant differences in the proportions reporting randomization were noted ($P<0.0001$, range 5.6%–46.5%) with a lack of randomization predominating in all cases except stroke (Figure 2A). Significant differences in the proportions of studies reporting randomization were also observed when stratified by animal model used ($P<0.0001$, range 14.6%–45.0%) with a lack of randomization predominating in all cases except when pigs or a combination of animal models were used (Figure 2B).

Blinding

Blinding of treatment allocation or outcome assessment was reported in 1110 studies (32.7%) overall, but was also more frequently noted in the subset examining therapeutic interventions (41.9% versus 28.9%, $P<0.0001$). When stratified by CVD studied, significant differences in the proportions of studies reporting any blinding were observed ($P<0.0001$, range 10.9%–62.6%). Studies on stroke ranked highest among CVDs although arterial aneurysms/dissections and transplantation medicine had comparable numbers of blinded and nonblinded studies. For all other CVDs, studies with blinding formed the minority (Figure 3A). No significant difference in the proportions of studies reporting blinding were seen when stratified by animal model used ($P=0.369$, range 20.9%–36.8%; Figure 3B).

Table. Study Characteristics

	Circulation (n=672)	*Circ Res* (n=486)	*Hypertension* (n=860)	*Stroke* (n=501)	*ATVB* (n=877)	Total (n=3396)
Disease studied						
Atherosclerosis	119 (17.7)	113 (23.3)	14 (1.6)	2 (0.4)	385 (43.9)	633 (18.6)
Hypertension	34 (5.1)	35 (7.2)	577 (67.1)	1 (0.2)	14 (1.6)	661 (19.5)
Arterial aneurysm	16 (2.4)	21 (4.3)	15 (1.7)	24 (4.8)	62 (7.1)	138 (4.1)
Myocardial infarction	95 (14.1)	56 (11.5)	19 (2.2)	0 (0)	115 (13.1)	285 (8.4)
Cardiomyopathy/heart failure	173 (25.7)	154 (31.7)	135 (15.7)	0 (0)	21 (2.4)	483 (14.2)
Pulmonary hypertension	57 (8.5)	29 (6.0)	34 (4.0)	0 (0)	15 (1.7)	135 (4.0)
Arrhythmia	65 (9.7)	35 (7.2)	4 (0.5)	0 (0)	0 (0)	104 (3.1)
Valvular disease	9 (1.3)	4 (0.8)	0 (0)	0 (0)	14 (1.6)	27 (0.8)
Stroke	20 (3.0)	4 (0.8)	9 (1.1)	435 (86.8)	18 (2.1)	486 (14.3)
Transplantation medicine	28 (4.2)	15 (3.1)	0 (0)	31 (6.2)	32 (3.7)	106 (3.1)
Metabolic/endocrine disorders	19 (2.8)	10 (2.1)	23 (2.7)	2 (0.4)	75 (8.6)	129 (3.8)
Hematologic disorders	19 (2.8)	8 (1.7)	1 (0.1)	0 (0)	79 (9.0)	107 (3.2)
Other	18 (2.7)	2 (0.4)	4 (0.5)	6 (1.2)	47 (5.4)	102 (3.0)
Animal model(s) used						
Mouse	463 (68.9)	391 (80.5)	357 (41.5)	223 (44.5)	744 (84.8)	2178 (64.1)
Rat	84 (12.5)	44 (9.1)	443 (51.5)	237 (47.3)	63 (7.3)	872 (25.7)
Rabbit	10 (1.5)	15 (3.1)	16 (1.9)	18 (3.6)	23 (2.6)	82 (2.4)
Dog	37 (5.5)	12 (2.5)	13 (1.5)	5 (1.0)	0 (0)	67 (2.0)
Pig	84 (12.5)	4 (0.8)	9 (1.1)	3 (0.6)	19 (2.2)	60 (1.8)
Primate	3 (0.5)	1 (0.2)	2 (0.2)	3 (0.6)	8 (0.9)	17 (0.5)
Other	10 (1.5)	4 (0.8)	9 (1.1)	1 (0.2)	1 (0.1)	25 (0.7)
Combination	40 (6.0)	15 (3.1)	11 (1.3)	11 (2.2)	18 (2.1)	95 (2.8)
Therapeutic study	162 (24.1)	85 (17.5)	221 (25.7)	264 (52.7)	256 (29.2)	988 (29.1)

Values reported as number (%). *ATVB* indicates *Arteriosclerosis, Thrombosis, and Vascular Biology*; and *Circ Res, Circulation Research*.

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Sample Size Estimation

A priori sample size estimations or power calculations were reported in 79 studies (2.3%). This aspect of study design was also more frequently reported in the subset examining therapeutic interventions (4.2% versus 1.6%, $P<0.0001$). Significant differences in the proportion of studies reporting sample size calculations were observed when stratified by CVD studied ($P<0.0001$, range 0%–9.5%) with stroke ranking highest (Figure 4A), but not when stratified by animal model used ($P=0.270$, range 0%–6.1%; Figure 4B).

Inclusion of Both Sexes

Sex bias prevalence and detailed temporal trends have been described previously.[29] After excluding studies that did not report the sex of the animals used, significant differences were noted in the proportions including animals of both sexes when stratified by CVD of interest ($P<0.0001$, range 2.4%–34.7%) and by animal model used ($P<0.0001$, range 6.7%–26.9%; Online Figure).

Temporal Patterns in Study Design Element Implementation

Over the past 10 years, there have been no significant changes in the proportions of studies reporting blinding or randomization (inclusion of both sexes has been reported previously[29]). There has been a significant increase in the proportion reporting a priori sample size estimations ($P_{trend}<0.0001$), although it remains below 7% (Figure 5). There was no substantial difference in the prevalence of any of these study design elements before and after the NIH principles and guidelines for reporting preclinical research were published in 2014 (range of differences 0.7%–3.6%). Post hoc calculations suggest that our sample sizes had ≥80% power to detect a difference of 5.0% for all study design elements.

CVD-specific temporal analyses suggest that preclinical studies on stroke are increasingly incorporating randomization, blinding, sample size estimations, and inclusion of both sexes—a pattern that is not seen in studies on atherosclerosis, hypertension, or cardiomyopathy/heart failure (the most

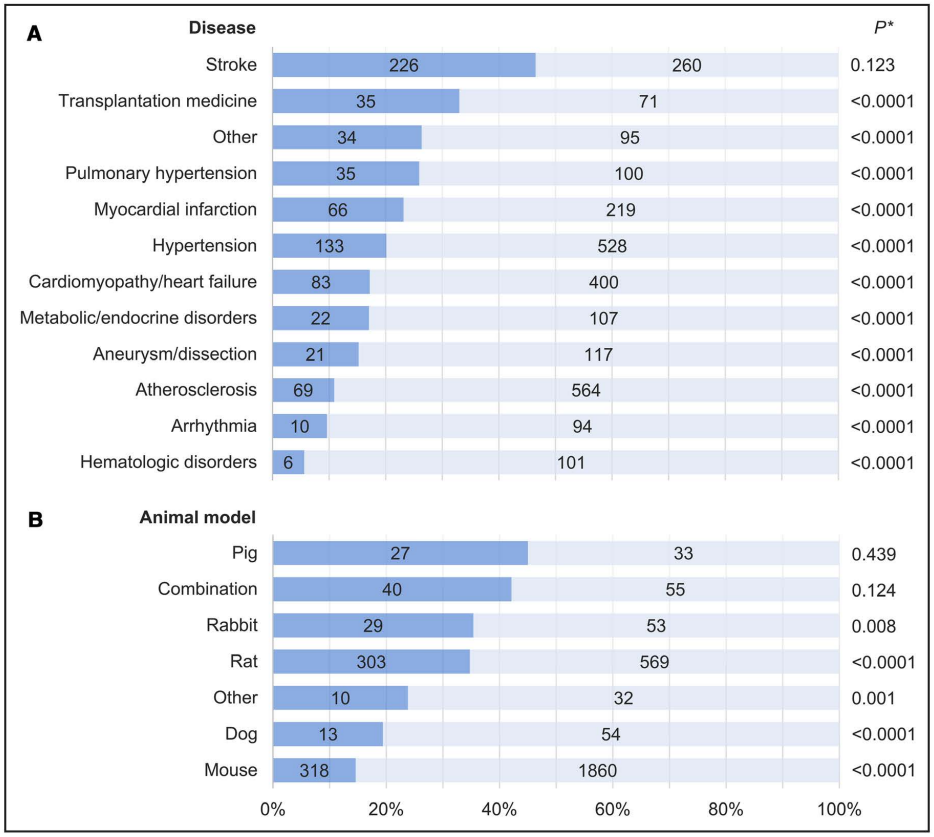


Figure 2. Randomization in preclinical cardiovascular research studies published over a 10-y period stratified by (A) disease studied and (B) species used. Dark blue corresponds to the proportion of studies implementing randomization; numbers in bars correspond to absolute numbers of studies. Valvular disease and resuscitative medicine studies were included in the "Other" category because of the small number of relevant publications. "Combination" refers to more than one animal species used within the same publication; animal models in the "Other" category included guinea pig, gerbil, and hamster. *For comparison of studies incorporating randomization vs not.

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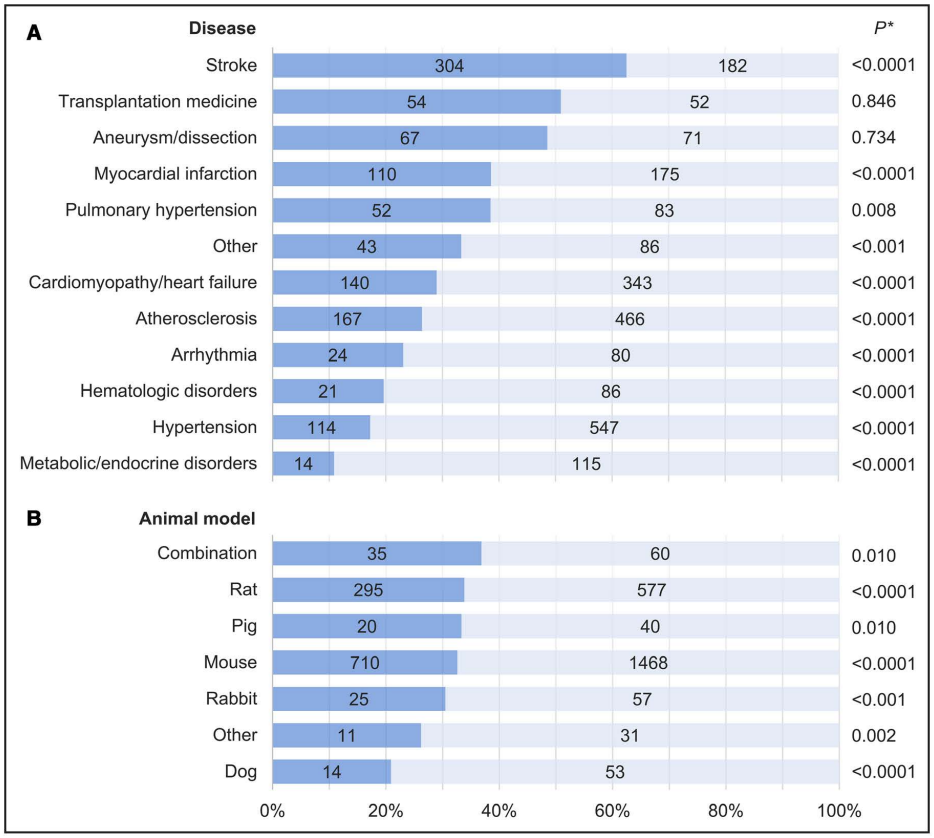


Figure 3. Blinding in preclinical cardiovascular research studies published over a 10-y period stratified by (A) disease studied and (B) species used. Dark blue corresponds to the proportion of studies implementing blinding; numbers in bars correspond to absolute numbers of studies. Valvular disease and resuscitative medicine studies were included in the "Other" category because of the small number of relevant publications. "Combination" refers to more than one animal species used within the same publication; animal models in the "Other" category included guinea pig, gerbil, and hamster. *For comparison of studies incorporating blinding vs not.

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commonly studied CVDs). Studies on atherosclerosis have shown a modest increase in the proportion reporting sample size estimation in recent years, but an overall significant decrease in the proportion reporting blinding, whereas the proportion of studies on cardiomyopathy/heart failure that include both sexes has significantly decreased. No other significant changes were observed during the 10-year period (Figure 6).

Given the disproportionate amount of stroke research published in *Stroke*, which has reported improvements in the quality and design of published preclinical research following the introduction of their "Basic Science Checklist" in 2011,[32] CVD-adjusted and animal model-adjusted comparisons of study design implementation before and after this time point were performed for each journal. *Stroke* uniquely exhibited significant improvements in all measures of methodological quality (range of adjusted odds ratios 2.4–8.2, $P<0.0001$ for all study design elements; Online Table). These analyses also identified stroke as the CVD studied as an independent positive predictor of one or more study design element in every journal.

Citations According to Index Study Methodology

At 60 months, 41441 articles citing the 1681 preclinical studies that were published between July 2006 and June 2011 were identified. The median citation count per preclinical study was 20 (interquartile range 13–31, range 0–131). Studies that implemented randomization, blinding, or sample size estimation had similar numbers of citations as those that did not; however, studies that included both males and females were cited less frequently (median 18 versus 20, $P=0.023$). The cumulative number of these study design elements was not associated with citation counts (Figure 7). No study implemented all 4 of the above design elements and only 20 studies (1.2%) incorporated 3; therefore, studies with ≥2 were grouped together for this analysis. The above associations (or lack thereof) between study design element(s) and number of citations persisted after adjusting for journal of publication, year of publication,

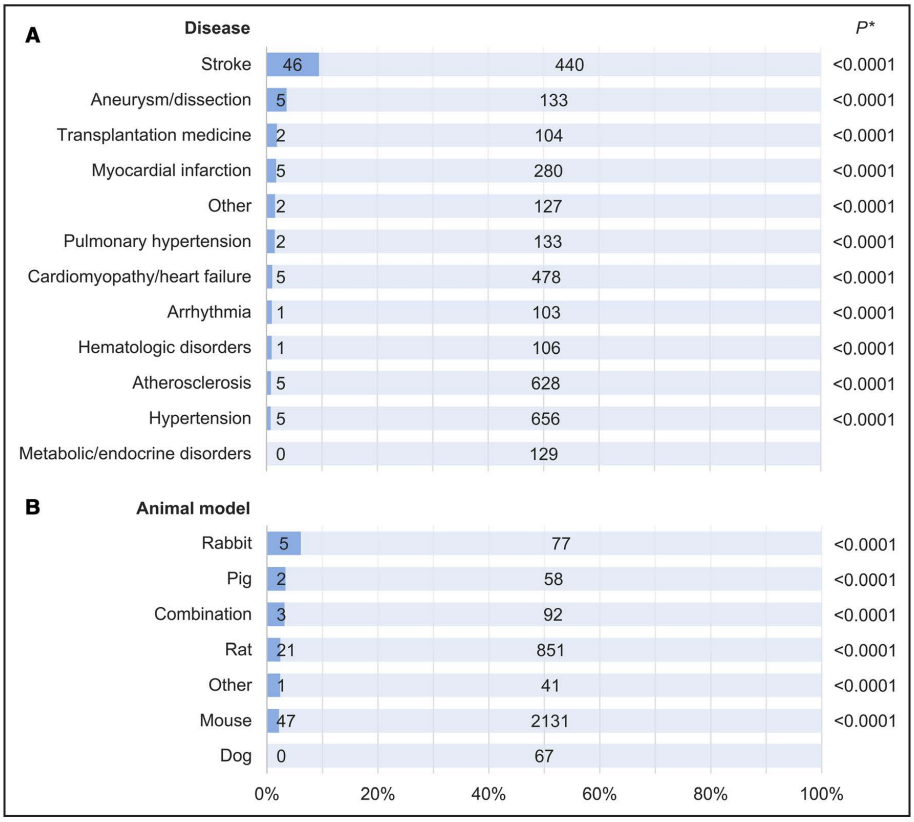


Figure 4. Sample size estimation in preclinical cardiovascular research studies published over a 10-y period stratified by (A) disease studied and (B) species used. Dark blue corresponds to the proportion of studies reporting sample size estimations/power calculations; numbers in bars correspond to absolute numbers of studies. Valvular disease and resuscitative medicine studies were included in the "Other" category because of the small number of relevant publications. "Combination" refers to more than one animal species used within the same publication; animal models in the "Other" category included guinea pig, gerbil, and hamster. *For comparison of studies incorporating sample size estimation vs not.

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and CVD studied in multivariable regression models and all findings were comparable in sensitivity analyses of citation counts at 36 months.

Discussion

Preclinical cardiovascular research using animal models plays an integral role in advancing the care of patients afflicted with CVDs. However, its impact is contingent on its scientific validity, reproducibility, and relevance to human physiology and disease. It is understood that inherent limitations of using animals to model human diseases can undermine the predictive value of preclinical findings, rendering it difficult for even the most skilled scientists to make impactful discoveries.[12] However, this difficulty is compounded when methodological sources of bias are introduced. We systematically examined a continuous and large body of preclinical cardiovascular research to determine how often randomization, blinding, and a priori sample size estimation are incorporated and did so over a sufficiently long period to draw meaningful conclusions on the temporal patterns of these practices. We report that, overall, these design elements are rarely implemented in studies published in leading peer-reviewed cardiovascular journals, paralleling the previously reported prevalence of sex bias.[29] Furthermore, apart from a modest increase in the proportion of studies reporting sample size calculations, there has been no improvement in these practices over the past decade, although stroke research may be a notable exception. Finally, analyses of citation counts suggest that crucial methodological aspects of preclinical studies may be overlooked by cardiovascular researchers, affording potentially biased research comparable influence on scientific research efforts as methodologically more robust studies.

Poorly designed preclinical studies not only contribute to experimental irreproducibility[2,7] and wasted resources[43,44] but also may result in erroneous conclusions regarding the treatment effects,[9,21,23,45,46] which can ultimately spur or deter clinical trials in humans with consequent risk of harm.[12,20,47] The experimental design elements evaluated in our study are

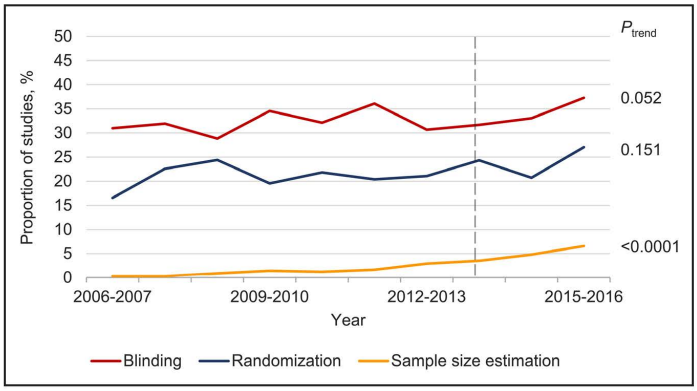


Figure 5. Temporal patterns in randomization, blinding, and sample size estimation in preclinical cardiovascular studies. Dashed line indicates the publication of the National Institutes of Health guidelines and policies for reporting preclinical research. Data for inclusion of both sexes have been reported previously.[29]

deemed crucial to improving the quality of preclinical research by addressing selection bias (randomization); minimizing performance, detection, and attrition bias (blinding); ensuring adequate statistical power and the ethical use of animals (sample size estimation); and promoting research relevance for both men and women (inclusion of both sexes).[1,6,7,10,13,26,48,49] Although these elements are routinely implemented in clinical trials as they have been shown to protect against bias and imprecision,[50–53] they have conspicuously not permeated preclinical experiments.[1,23,54–56] The high degree of experimental control that is possible in preclinical research (eg, via genetic and environmental homogeneity) can reduce variation and the sample sizes required relative to clinical trials; however, variations in injury or disease induction and the potential for persistent and unrecognized confounders represent important sources of bias.[6,31,57]

Furthermore, despite a pervasive belief among scientists that there is a reproducibility "crisis" in research that is attributable to methodological and reporting shortcomings,[14] our citation analyses suggest that greater methodological rigor in preclinical cardiovascular research does not translate into greater scientific influence. A similar observation was noted by Amgen in a study of 53 preclinical cancer research publications: studies that they could not adequately reproduce were cited as often, if not more often, than those that they could successfully reproduce, irrespective of the journal of

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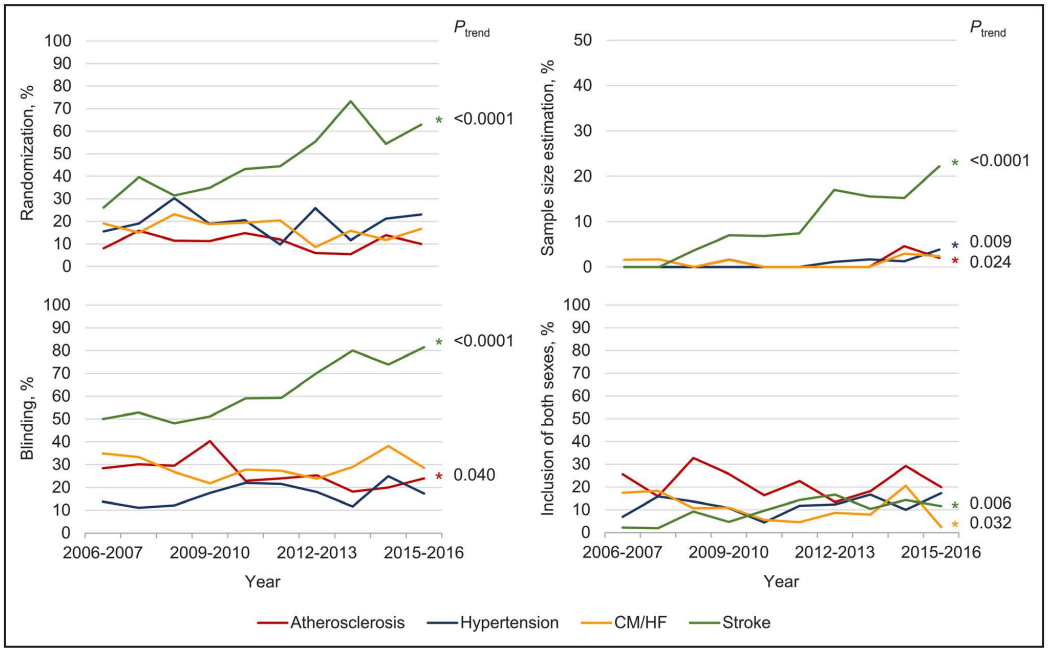


Figure 6. Temporal patterns in randomization, blinding, sample size estimation, and inclusion of both sexes in preclinical research for the most commonly studied cardiovascular diseases. Note the different scale of *y*-axis for sample size estimation. CM/HF indicates cardiomyopathy/heart failure.

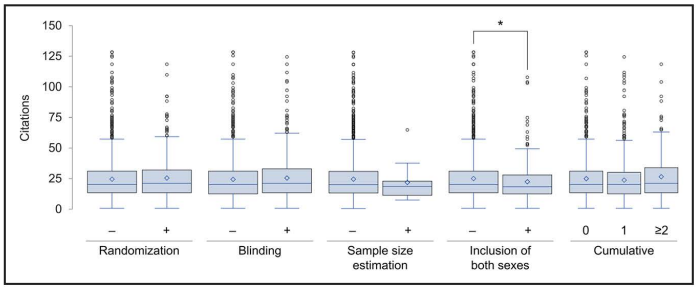


Figure 7. Box-whisker plot of the number of citations by methodological rigor of index preclinical study (n=1681). Diamond symbol plotted at mean. Outliers identified as beyond 1.5 interquartile range. Cumulative refers to sum of randomization, blinding, sample size estimation, and inclusion of both sexes (each contributing 1 point). *P<0.05.

publication's impact factor.[12] The suggestion that methodologically robust studies are given equal consideration as studies at greater risk of bias by cardiovascular researchers is troubling as it undermines the reputed "self-correcting" tenet of science and increases the risk of pursuing unfruitful avenues of research.[2,5,44,48]

Cogent analyses and arguably practical solutions to improve preclinical research quality (and secondarily to enhance research translation) have been proposed. However, evidence of changes in research practice and data on the impact of corrective actions are scarce.[13] Several guidelines and checklists have been developed to improve preclinical methodology[45,58,59] and reporting,[25,45,60–62] yet there is little indication that they are effecting change despite being widely endorsed.[43,58,63] Systematic reviews and meta-analyses have been advocated as safeguards to expose bias in preclinical research before embarking on clinical trials,[9,47,64,65] but they are limited by the internal validity of included studies[66] and are not routinely performed. Changes in research funding such as those undertaken by the NIH[2,26] may bolster these efforts; however, our analyses of animal studies in the cardiovascular sciences have not shown signs of substantial changes in research practices or reporting since they were announced.[29]

Journal editors and reviewers directly influence what is published thereby often serving as ultimate gatekeepers of research findings; yet, few journals effectively encourage the use of reporting guidelines.[67] At the time of writing, there exists considerable variation in author guidelines for reporting animal studies among leading cardiovascular journals with few requiring authors to report randomization, blinding, sample size estimation, or the sex of the animals used—requirements that could increase transparency and reinforce the importance of these study design elements.[13] Uniquely, however, *Stroke* implemented a "Basic Science Checklist" in 2011 (updated in 2016),[28] which includes all of these relevant elements, forms part of the manuscript submission process, and is evaluated by editors and reviewers. Over the 2.5 years after its introduction, Minnerup et al[32] noted improvements in randomization, blinding, and allocation concealment compared with the preceding 18 months. We noted significant improvements in the quality of preclinical stroke research, 90% of which was published in *Stroke*, raising the question of whether these improvements were due to journal editorial policies/culture or driven by changes in research practices among stroke researchers in general. Our post hoc analyses suggest that it is likely a combination of both: *Stroke* uniquely showed improvements in all study design elements even after adjusting for CVD studied and animal model used, but stroke as the CVD studied was identified as an independent predictor for at least one study design element for every journal examined. Our findings, therefore, corroborate those of Minnerup et al[32] and expand upon them by demonstrating that these improvements have continued into 2016, extend to sample size estimation (and to a lesser extent inclusion of both sexes), contrast with an extended preceding period of suboptimal reporting, and have not been paralleled in other prominent cardiovascular journals. Therefore, although not conclusive, our data suggest that journal editors and reviewers can exert considerable influence on preclinical research practices and reporting. Yet, our findings also suggest that stroke research has independently improved in quality—a finding that may be attributable to its community's early appreciation of the importance of methodological rigor in preclinical science and extensive involvement in efforts to improve research translation.[7,20,21,46,58,68–71]

Our study is not without limitations. The study sample comprised articles from 5 cardiovascular journals over 10 years, which may not fully represent preclinical cardiovascular research publications or practices. However, we deliberately selected AHA journals given their prominence in the field of cardiovascular medicine, established reputation, collective focus on a broad range of CVDs, and unanimous endorsement of the NIH guidelines on rigor and reproducibility. Given that none of the American College of Cardiology or European Society of Cardiology journals have endorsed the guidelines so far,[25] our data may actually overestimate the methodological quality of most preclinical cardiovascular research. There is no accepted definition for preclinical studies therefore criteria were developed for this study. It is, therefore, possible that not all relevant studies were included in our analysis. However, the inclusion criteria used, the number of journals reviewed, and the substantial inter-rater agreement for study inclusion support the validity of our results and render our data the best available on methodological rigor in preclinical cardiovascular research. Furthermore, our inclusion criteria are in line with previously proposed distinctions between "exploratory" and "confirmatory" preclinical research.[7,30,31] Our analysis considered blinding as a single and dichotomous variable, which limits detailed assessments of this design element, and did not examine all relevant potential sources of bias. Data handling and publication bias, for instance, are important factors that may contribute to irreproducibility in science.[2,6,7,9–11,13,43] As well, factors other than experimental

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methodology can influence the predictive value of preclinical research, including how well an animal model reflects human physiology and disease,[6,9,45,72] the appropriateness of statistical analyses,[73] and a lack of standardization of definitions and surrogate markers,[74,75] which were not examined. Although article citation is the most commonly used measure of research impact, it is an imperfect indicator.[33,34] Finally, study design elements may have been implemented but not reported, which could result in underestimates of their prevalence. However, underreporting of measures of methodological rigor is believed to be low[49,65] and therefore unlikely to have significantly influenced our results.

Our analysis of preclinical research published in leading cardiovascular journals over the past 10 years demonstrates that methodological sources of potential bias and imprecision are prevalent, have not appreciably improved over time, and may be overlooked by researchers when basing subsequent studies. Concerted efforts to address this problem are urgently needed. Stroke research has uniquely shown substantial improvement in several measures of quality in recent years, warranting a closer examination to identify drivers of its success.

Disclosures

None.

References

1. Ioannidis JP, Greenland S, Hlatky MA, Khoury MJ, Macleod MR, Moher D, Schulz KF, Tibshirani R. Increasing value and reducing waste in research design, conduct, and analysis. *Lancet*. 2014;383:166–175. doi: 10.1016/S0140-6736(13)62227-8.
2. Collins FS, Tabak LA. Policy: NIH plans to enhance reproducibility. *Nature*. 2014;505:612–613.
3. Henderson VC, Kimmelman J, Fergusson D, Grimshaw JM, Hackam DG. Threats to validity in the design and conduct of preclinical efficacy studies: a systematic review of guidelines for in vivo animal experiments. *PLoS Med*. 2013;10:e1001489. doi: 10.1371/journal.pmed.1001489.
4. Pammolli F, Magazzini L, Riccaboni M. The productivity crisis in pharmaceutical R&D. *Nat Rev Drug Discov*. 2011;10:428–438. doi: 10.1038/nrd3405.
5. Bunnage ME. Getting pharmaceutical R&D back on target. *Nat Chem Biol*. 2011;7:335–339. doi: 10.1038/nchembio.581.
6. van der Worp HB, Howells DW, Sena ES, Porritt MJ, Rewell S, O'Collins V, Macleod MR. Can animal models of disease reliably inform human studies? *PLoS Med*. 2010;7:e1000245. doi: 10.1371/journal.pmed.1000245.
7. Landis SC, Amara SG, Asadullah K, et al. A call for transparent reporting to optimize the predictive value of preclinical research. *Nature*. 2012;490:187–191. doi: 10.1038/nature11556.
8. Scott S, Kranz JE, Cole J, Lincecum JM, Thompson K, Kelly N, Bostrom A, Theodoss J, Al-Nakhala BM, Vieira FG, Ramasubbu J, Heywood JA. Design, power, and interpretation of studies in the standard murine model of ALS. *Amyotroph Lateral Scler*. 2008;9:4–15. doi: 10.1080/17482960701856300.
9. Perel P, Roberts I, Sena E, Wheble P, Briscoe C, Sandercock P, Macleod M, Mignini LE, Jayaram P, Khan KS. Comparison of treatment effects between animal experiments and clinical trials: systematic review. *BMJ*. 2007;334:197. doi: 10.1136/bmj.39048.407928.BE.
10. Muhlhausler BS, Bloomfield FH, Gillman MW. Whole animal experiments should be more like human randomized controlled trials. *PLoS Biol*. 2013;11:e1001481. doi: 10.1371/journal.pbio.1001481.
11. Prinz F, Schlange T, Asadullah K. Believe it or not: how much can we rely on published data on potential drug targets? *Nat Rev Drug Discov*. 2011;10:712. doi: 10.1038/nrd3439-c1.
12. Begley CG, Ellis LM. Drug development: Raise standards for preclinical cancer research. *Nature*. 2012;483:531–533. doi: 10.1038/483531a.
13. Begley CG, Ioannidis JP. Reproducibility in science: improving the standard for basic and preclinical research. *Circ Res*. 2015;116:116–126. doi: 10.1161/CIRCRESAHA.114.303819.
14. Baker M. 1,500 scientists lift the lid on reproducibility. *Nature*. 2016;533:452–454. doi: 10.1038/533452a.
15. Unreliable research: Trouble at the lab. *The Economist*. 2013.
16. Lehrer J. The truth wears off: Is there something wrong with the scientific method? *The New Yorker*. 2010.
17. Freedman DH. Lies, damned lies, and medical science. *The Atlantic*. 2010.
18. Osherovich L. Hedging against academic risk. *Science-Business eXchange*. 2011;4:doi:10.1038/scibx.2011.1416
19. Begley CG. Six red flags for suspect work. *Nature*. 2013;497:433–434. doi: 10.1038/497433a.
20. Horn J, de Haan RJ, Vermeulen M, Luiten PG, Limburg M. Nimodipine in animal model experiments of focal cerebral ischemia: a systematic review. *Stroke*. 2001;32:2433–2438.
21. Macleod MR, van der Worp HB, Sena ES, Howells DW, Dirnagl U, Donnan GA. Evidence for the efficacy of NXY-059 in experimental focal cerebral ischaemia is confounded by study quality. *Stroke*. 2008;39:2824–2829. doi: 10.1161/STROKEAHA.108.515957.
22. Ioannidis JP. Why most published research findings are false. *PLoS Med*. 2005;2:e124. doi: 10.1371/journal.pmed.0020124.
23. Hackam DG, Redelmeier DA. Translation of research evidence from animals to humans. *JAMA*. 2006;296:1731–1732. doi: 10.1001/jama.296.14.1731.
24. Hay M, Thomas DW, Craighead JL, Economides C, Rosenthal J. Clinical development success rates for investigational drugs. *Nat Biotechnol*. 2014;32:40–51. doi: 10.1038/nbt.2786.
25. National Institutes of Health. Principles and guidelines for reporting preclinical research. https://www.nih.gov/research-training/rigor-reproducibility/principles-guidelines-reporting-preclinical-research. Accessed December 2, 2016.
26. Clayton JA, Collins FS. Policy: NIH to balance sex in cell and animal studies. *Nature*. 2014;509:282–283.
27. Daugherty A, Hegele RA, Mackman N, Rader DJ, Schmidt AM, Weber C. Complying with the national institutes of health guidelines and principles for rigor and reproducibility: refutations. *Arterioscler Thromb Vasc Biol*. 2016;36:1303–1304. doi: 10.1161/ATVBAHA.116.307906.
28. Vahidy F, Schäbitz WR, Fisher M, Aronowski J. Reporting standards for preclinical studies of stroke therapy. *Stroke*. 2016;47:2435–2438. doi: 10.1161/STROKEAHA.116.013643.
29. Ramirez FD, Motazedian P, Jung RG, Di Santo P, MacDonald Z, Simard T, Clancy AA, Russo JJ, Welch V, Wells GA, Hibbert B. Sex bias is increasingly prevalent in preclinical cardiovascular research: implications for translational medicine and health equity for women: a systematic assessment of leading cardiovascular journals over a 10-year period. *Circulation*. 2017;135:625–626. doi: 10.1161/CIRCULATIONAHA.116.026668.
30. Kimmelman J, Mogil JS, Dirnagl U. Distinguishing between exploratory and confirmatory preclinical research will improve translation. *PLoS Biol*. 2014;12:e1001863. doi: 10.1371/journal.pbio.1001863.
31. Unger EF. All is not well in the world of translational research. *J Am Coll Cardiol*. 2007;50:738–740. doi: 10.1016/j.jacc.2007.04.067.
32. Minnerup J, Zentsch V, Schmidt A, Fisher M, Schäbitz WR. Methodological quality of experimental stroke studies published in the stroke journal: time trends and effect of the basic science checklist. *Stroke*. 2016;47:267–272. doi: 10.1161/STROKEAHA.115.011695.
33. Ranasinghe I, Shojaee A, Bikdeli B, Gupta A, Chen R, Ross JS, Masoudi FA, Spertus JA, Nallamothu BK, Krumholz HM. Poorly cited articles in peer-reviewed cardiovascular journals from 1997 to 2007: analysis of 5-year citation rates. *Circulation*. 2015;131:1755–1762. doi: 10.1161/CIRCULATIONAHA.114.015080.
34. Danthi N, Wu CO, Shi P, Lauer M. Percentile ranking and citation impact of a large cohort of National Heart, Lung, and Blood Institute-funded cardiovascular R01 grants. *Circ Res*. 2014;114:600–606. doi: 10.1161/CIRCRESAHA.114.302656.
35. Kaltman JR, Evans FJ, Danthi NS, Wu CO, DiMichele DM, Lauer MS. Prior publication productivity, grant percentile ranking, and topic-normalized citation impact of NHLBI cardiovascular R01 grants. *Circ Res*. 2014;115:617–624. doi: 10.1161/CIRCRESAHA.115.304766.
36. Lauer MS, Danthi NS, Kaltman J, Wu C. Predicting productivity returns on investment: thirty years of peer review, grant funding, and publication of highly cited papers at the National Heart, Lung, and Blood Institute. *Circ Res*. 2015;117:239–243. doi: 10.1161/CIRCRESAHA.115.306830.
37. Fabsitz RR, Papanicolaou GJ, Sholinsky P, Coady SA, Jaquish CE, Nelson CR, Olson JL, Puggal MA, Purkiser KL, Srinivas PR, Wei GS, Wolz M, Sorlie PD. Impact of National Heart, Lung, and Blood Institute-Supported Cardiovascular Epidemiology Research, 1998 to 2012. *Circulation*. 2015;132:2028–2033. doi: 10.1161/CIRCULATIONAHA.114.014147.

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38. Kulkarni AV, Aziz B, Shams I, Busse JW. Comparisons of citations in Web of Science, Scopus, and Google Scholar for articles published in general medical journals. *JAMA*. 2009;302:1092–1096. doi: 10.1001/jama.2009.1307.
39. Steen RG, Casadevall A, Fang FC. Why has the number of scientific retractions increased? *PLoS One*. 2013;8:e68397. doi: 10.1371/journal.pone.0068397.
40. Concato J, Peduzzi P, Holford TR, Feinstein AR. Importance of events per independent variable in proportional hazards analysis. I. Background, goals, and general strategy. *J Clin Epidemiol*. 1995;48:1495–1501.
41. Peduzzi P, Concato J, Feinstein AR, Holford TR. Importance of events per independent variable in proportional hazards regression analysis. II. Accuracy and precision of regression estimates. *J Clin Epidemiol*. 1995;48:1503–1510.
42. Peduzzi P, Concato J, Kemper E, Holford TR, Feinstein AR. A simulation study of the number of events per variable in logistic regression analysis. *J Clin Epidemiol*. 1996;49:1373–1379.
43. Glasziou P, Altman DG, Bossuyt P, Boutron I, Clarke M, Julious S, Michie S, Moher D, Wager E. Reducing waste from incomplete or unusable reports of biomedical research. *Lancet*. 2014;383:267–276. doi: 10.1016/S0140-6736(13)62228-X.
44. Freedman LP, Cockburn IM, Simcoe TS. The economics of reproducibility in preclinical research. *PLoS Biol*. 2015;13:e1002165. doi: 10.1371/journal.pbio.1002165.
45. Macleod MR, O'Collins T, Howells DW, Donnan GA. Pooling of animal experimental data reveals influence of study design and publication bias. *Stroke*. 2004;35:1203–1208. doi: 10.1161/01.STR.0000125719.25853.20.
46. Macleod MR, O'Collins T, Horky LL, Howells DW, Donnan GA. Systematic review and metaanalysis of the efficacy of FK506 in experimental stroke. *J Cereb Blood Flow Metab*. 2005;25:713–721. doi: 10.1038/sj.jcbfm.9600064.
47. Pound P, Ebrahim S, Sandercock P, Bracken MB, Roberts I; Reviewing Animal Trials Systematically (RATS) Group. Where is the evidence that animal research benefits humans? *BMJ*. 2004;328:514–517. doi: 10.1136/bmj.328.7438.514.
48. Clayton JA. Studying both sexes: a guiding principle for biomedicine. *FASEB J*. 2016;30:519–524. doi: 10.1096/fj.15-279554.
49. Macleod MR, Lawson McLean A, Kyriakopoulou A, et al. Risk of bias in reports of in vivo research: a focus for improvement. *PLoS Biol*. 2015;13:e1002273. doi: 10.1371/journal.pbio.1002273.
50. Savović J, Jones HE, Altman DG, et al. Influence of reported study design characteristics on intervention effect estimates from randomized, controlled trials. *Ann Intern Med*. 2012;157:429–438. doi: 10.7326/0003-4819-157-6-201209180-00537.
51. Tzoulaki I, Siontis KC, Ioannidis JP. Prognostic effect size of cardiovascular biomarkers in datasets from observational studies versus randomised trials: meta-epidemiology study. *BMJ*. 2011;343:d6829.
52. Lijmer JG, Mol BW, Heisterkamp S, Bonsel GJ, Prins MH, van der Meulen JH, Bossuyt PM. Empirical evidence of design-related bias in studies of diagnostic tests. *JAMA*. 1999;282:1061–1066.
53. Rutjes AW, Reitsma JB, Di Nisio M, Smidt N, van Rijn JC, Bossuyt PM. Evidence of bias and variation in diagnostic accuracy studies. *CMAJ*. 2006;174:469–476. doi: 10.1503/cmaj.050090.
54. Kilkenny C, Parsons N, Kadyszewski E, Festing MF, Cuthill IC, Fry D, Hutton J, Altman DG. Survey of the quality of experimental design, statistical analysis and reporting of research using animals. *PLoS One*. 2009;4:e7824. doi: 10.1371/journal.pone.0007824.
55. Beery AK, Zucker I. Sex bias in neuroscience and biomedical research. *Neurosci Biobehav Rev*. 2011;35:565–572. doi: 10.1016/j.neubiorev.2010.07.002.
56. Yoon DY, Mansukhani NA, Stubbs VC, Helenowski IB, Woodruff TK, Kibbe MR. Sex bias exists in basic science and translational surgical research. *Surgery*. 2014;156:508–516. doi: 10.1016/j.surg.2014.07.001.
57. Reardon S. A mouse's house may ruin experiments. *Nature*. 2016;530:264. doi: 10.1038/nature.2016.19335.
58. Fisher M, Feuerstein G, Howells DW, Hurn PD, Kent TA, Savitz SI, Lo EH; STAIR Group. Update of the stroke therapy academic industry roundtable preclinical recommendations. *Stroke*. 2009;40:2244–2250. doi: 10.1161/STROKEAHA.108.541128.
59. Hooijmans CR, Rovers MM, de Vries RB, Leenaars M, Ritskes-Hoitinga M, Langendam MW. SYRCLE's risk of bias tool for animal studies. *BMC Med Res Methodol*. 2014;14:43. doi: 10.1186/1471-2288-14-43.
60. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol*. 2010;8:e1000412. doi: 10.1371/journal.pbio.1000412.
61. Announcement: Reducing our irreproducibility. *Nature*. 2013;496
62. Hooijmans CR, Leenaars M, Ritskes-Hoitinga M. A gold standard publication checklist to improve the quality of animal studies, to fully integrate the Three Rs, and to make systematic reviews more feasible. *Altern Lab Anim*. 2010;38:167–182.
63. Baker D, Lidster K, Sottomayor A, Amor S. Two years later: journals are not yet enforcing the ARRIVE guidelines on reporting standards for preclinical animal studies. *PLoS Biol*. 2014;12:e1001756. doi: 10.1371/journal.pbio.1001756.
64. Sandercock P, Roberts I. Systematic reviews of animal experiments. *Lancet*. 2002;360:586. doi: 10.1016/S0140-6736(02)09812-4.
65. Hirst JA, Howick J, Aronson JK, Roberts N, Perera R, Koshiaris C, Heneghan C. The need for randomization in animal trials: an overview of systematic reviews. *PLoS One*. 2014;9:e98856. doi: 10.1371/journal.pone.0098856.
66. van Luijk J, Bakker B, Rovers MM, Ritskes-Hoitinga M, de Vries RB, Leenaars M. Systematic reviews of animal studies; missing link in translational research? *PLoS One*. 2014;9:e89981. doi: 10.1371/journal.pone.0089981.
67. Hirst A, Altman DG. Are peer reviewers encouraged to use reporting guidelines? A survey of 116 health research journals. *PLoS One*. 2012;7:e35621. doi: 10.1371/journal.pone.0035621.
68. Stroke Therapy Academic Industry Roundtable (STAIR). Recommendations for standards regarding preclinical neuroprotective and restorative drug development. *Stroke*. 1999;30:2752–2758
69. O'Collins VE, Macleod MR, Donnan GA, Horky LL, van der Worp BH, Howells DW. 1,026 experimental treatments in acute stroke. *Ann Neurol*. 2006;59:467–477. doi: 10.1002/ana.20741.
70. Crossley NA, Sena E, Goehler J, Horn J, van der Worp B, Bath PM, Macleod M, Dirnagl U. Empirical evidence of bias in the design of experimental stroke studies: a metaepidemiologic approach. *Stroke*. 2008;39:929–934. doi: 10.1161/STROKEAHA.107.498725.
71. Sena E, van der Worp HB, Howells D, Macleod M. How can we improve the pre-clinical development of drugs for stroke? *Trends Neurosci*. 2007;30:433–439. doi: 10.1016/j.tins.2007.06.009.
72. Perrin S. Preclinical research: Make mouse studies work. *Nature*. 2014;507:423–425. doi: 10.1038/507423a.
73. García-Berthou E, Alcaraz C. Incongruence between test statistics and P values in medical papers. *BMC Med Res Methodol*. 2004;4:13. doi: 10.1186/1471-2288-4-13.
74. Johnson VE. Revised standards for statistical evidence. *Proc Natl Acad Sci U S A*. 2013;110:19313–19317. doi: 10.1073/pnas.1313476110.
75. Ioannidis JP. How to make more published research true. *PLoS Med*. 2014;11:e1001747. doi: 10.1371/journal.pmed.1001747.

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Appendix T

Progenitor cells for arterial repair: incremental advancements towards therapeutic reality

Citation information:

Jung RG*, Simard T*, Motazedian P, Di Santo P, Ramirez FD, Russo J, Labinaz A, Yousef A, Anantharam B, Pourdjabbar A, Hibbert B. Progenitor cells for arterial repair: incremental advancements towards therapeutic reality. **(2017)**. *Stem Cells International.* 2017:ID 8270498

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Hindawi Publishing Corporation
Stem Cells International
Volume 2017, Article ID 8270498, 14 pages
http://dx.doi.org/10.1155/2017/8270498

Review Article

Progenitor Cells for Arterial Repair: Incremental Advancements towards Therapeutic Reality

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Received 8 October 2016; Accepted 18 December 2016; Published 23 January 2017

Academic Editor: Rebecca D. Levit

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Coronary revascularization remains the standard treatment for obstructive coronary artery disease and can be accomplished by either percutaneous coronary intervention (PCI) or coronary artery bypass graft surgery. Considerable advances have rendered PCI the most common form of revascularization and improved clinical outcomes. However, numerous challenges to modern PCI remain, namely, in-stent restenosis and stent thrombosis, underscoring the importance of understanding the vessel wall response to injury to identify targets for intervention. Among recent promising discoveries, endothelial progenitor cells (EPCs) have garnered considerable interest given an increasing appreciation of their role in vascular homeostasis and their ability to promote vascular repair after stent placement. Circulating EPC numbers have been inversely correlated with cardiovascular risk, while administration of EPCs in humans has demonstrated improved clinical outcomes. Despite these encouraging results, however, advancing EPCs as a therapeutic modality has been hampered by a fundamental roadblock: what constitutes an EPC? We review current definitions and sources of EPCs as well as the proposed mechanisms of EPC-mediated vascular repair. Additionally, we discuss the current state of EPCs as therapeutic agents, focusing on endogenous augmentation and transplantation.

1. Introduction

Coronary artery disease (CAD) remains a leading cause of morbidity and mortality [1]. Coronary revascularization (restoring blood flow to the myocardium) remains the standard treatment for obstructive CAD and can be accomplished by either percutaneous coronary intervention (PCI) or coronary artery bypass graft (CABG) surgery. PCI has become the most frequently performed means of revascularization, aided by considerable advances in the field. What started as simple balloon angioplasty has now evolved through many generations of vascular scaffolds, from bare-metal stents (BMSs) to first and then second generation drug-eluting stents (DESs) [2–5]. While early technologies in PCI were fraught with high rates of complications including abrupt vessel closure, subsequent advances have mitigated much of this risk. Nevertheless, numerous challenges remain, including in-stent restenosis (ISR) and stent thrombosis (ST), collectively occurring in 5–8% of cases per year [6, 7]. Moreover, our understanding of the vessel wall's response to injury continues to evolve offering further opportunities for therapeutic intervention as our understanding of the impact that progenitor populations play has crystallized. Over the last decade, characterizing progenitor cell populations, understanding their role in vascular homeostasis, and harnessing their therapeutic potential by modifying them at a biological level remain areas of intense investigation.

The vessel wall's response to injury (e.g., plaque disruption and/or stent deployment) depends on many interdependent factors, including the insult itself in combination with the dynamic nature of the vessel wall-stent interface (including the cellular composition of the vessel wall and stent material and dimensions), vascular tone, and circulating signals. Indeed, PCI itself prompts endothelial denudation

and disruption of vascular homeostasis given the high pressure balloon inflation and subsequent force transmitted by stent struts to the endothelial layer extending damage through the underlying medial and adventitial layers [8]. In response to injury, the endothelium secretes a variety of molecules that influence vascular tone, inflammatory cell invasion, thrombus formation, and smooth muscle cell proliferation [9]. Indeed, stent-induced endothelial injury leads to platelet activation and thrombus propagation which alone can result in stent thrombosis [10]. As well, injury mediated inflammation is driven by activation of hypoxia-induced factor (HIF), which has a downstream effect on a number of factors including stromal cell derived factor-1 (SDF-1), angiopoietin, interleukin-8 (IL-8), vascular endothelial growth factor (VEGF), and c-Kit ligand to name a few [11]. Collectively, this activation stimulates the influx of inflammatory cells (neutrophils, monocytes, and macrophages) into the vascular wall, while also stimulating smooth muscle cells (SMCs) and myofibroblasts leading to neointima (NI) formation and subsequent ISR [12]. Paradoxically, this process, including VEGF secretion in particular, provides a stimulus for mobilizing endothelial progenitor cells (EPCs) to the site of injury and mediating vascular repair [13], the mechanisms of which are reviewed in detail below.

EPCs represent an attractive therapeutic option given their purported ability to promote vascular repair after stent placement. Accordingly, an inverse correlation between circulating EPC numbers and both atherosclerotic burden and probability of future cardiovascular risk has been noted [14, 15]. Furthermore, patients with ISR also have lower numbers of circulating EPCs and impaired EPC function [16, 17]. Given the potential benefits of EPC activity at sites of vascular injury, means of augmenting their number and/or function has been pursued and bolstered by clinical and animal studies suggesting improved outcomes following administration of EPCs [18, 19]. Furthermore, evidence suggests that EPCs are mobilized in patients with acute coronary syndromes, suggesting the presence of an endogenous mechanism that, if harnessed, could potentially improve outcomes [20, 21]. This work has stimulated interest in the importance of EPC signaling, number, and function in modulating vascular homeostasis with the potential for therapeutic benefit by capitalizing on EPC biology, particularly in the setting of PCI [22]. Herein, we review the biology of EPCs and their role as marker, modulator, and therapeutic agent for vascular repair.

2. Endothelial Progenitor Cells

Vascular progenitor cells were first observed in animal models when implanting Dacron grafts or silastic tubes [23–29]. These experiments first indicated the existence of a "pseudointima" composed primarily of endothelial cells and vascular smooth muscle cells, generating interest as to the origin of these cells [30]. This origin was finally discovered in 1997, when Asahara isolated EPCs from peripheral blood, describing them as CD34+ cells circulating in the human vasculature which were subsequently implicated in vascular homeostasis and endothelial repair [5, 31–34]. Subsequent studies described the incorporation of bone marrow-derived EPCs into blood vessels following hind limb ischemia [35] and myocardial infarction [36] as well as at wound [37] and tumour [38] sites. EPCs are believed to promote vascular repair by homing to sites of vascular injury where they act via [1] paracrine signaling to neighbouring cells (now believed to represent the predominant mode of action) and [2] trans-differentiating to mature endothelial cells and undergoing angiogenesis to form new blood vessels [5, 39] (Figure 1). In this way, EPCs are believed to hold therapeutic promise, though harnessing this potential has proven challenging.

2.1. The Evolving Definition of EPCs. Since their discovery, the precise definition of EPCs remains the subject of considerable debate and represents a major hindrance in understanding the biology and application of these unique cells. This is believed to be a driving force behind conflicting results noted in both preclinical and clinical trials, where differing methodologies in cell sources, cell purification, detection methods, and animal models or assays have been published [40]. Thus far, EPCs have been identified through two main methods: (1) cell-surface markers and (2) colony forming units (CFUs) and cultured/circulated angiogenic cells (CACs) (Figure 2). However, while these methods allow for the physical identification of EPCs, they are unable to characterize the in vitro activity of these cells nor the phenotype they express in physiologic conditions. We herein briefly discuss two common methods used to define EPCs.

2.1.1. Identification by Cell-Surface Markers. EPCs can be classified based on the expression of specific cell-surface antigens via flow cytometry (Table 1). Mature endothelial cells (ECs) express multiple surface antigens including CD34, VE-cadherin, von Willebrand factor (vWF), kinase insert domain-containing receptor (KDR) or VEGFR2, and E-selectin [41]. However, surface antigen expression varies between mature ECs depending on the specific vessel, organ, and activation state of the endothelium. To distinguish between EPCs and mature ECs shed after vascular injury, CD133 (AC133) was utilized, given that it is typically lost during maturation of EPCs into mature ECs. Surface markers CD34, VEGFR2, and CD133 have been widely accepted by researchers for defining EPCs, though many studies typically employ two of the three receptors [40, 41]. Other studies of EPCs have employed CD45dim/CD34/CD133/CD117, with CD117 playing a role in mobilization of hematopoietic stem cells [32, 42, 43]. In 2007, Case et al. isolated EPCs from adult peripheral blood by targeting CD34+ VEGFR2+ and CD133+ cells which did not contribute to the formation of mature endothelial cells in vitro, further challenging the existing models of progenitor cells [44]. Despite controversy, cell-surface marker-based definitions of EPCs (namely, CD34+/KDR+) have been demonstrated to be predictive of cardiovascular outcomes in patients, lending support to this approach [15]. Further work, including in vivo and in vitro assessment of endothelial differentiation capacity, is required to better define this subpopulation of progenitors and ensure future studies and therapies focus on a homogeneous population.

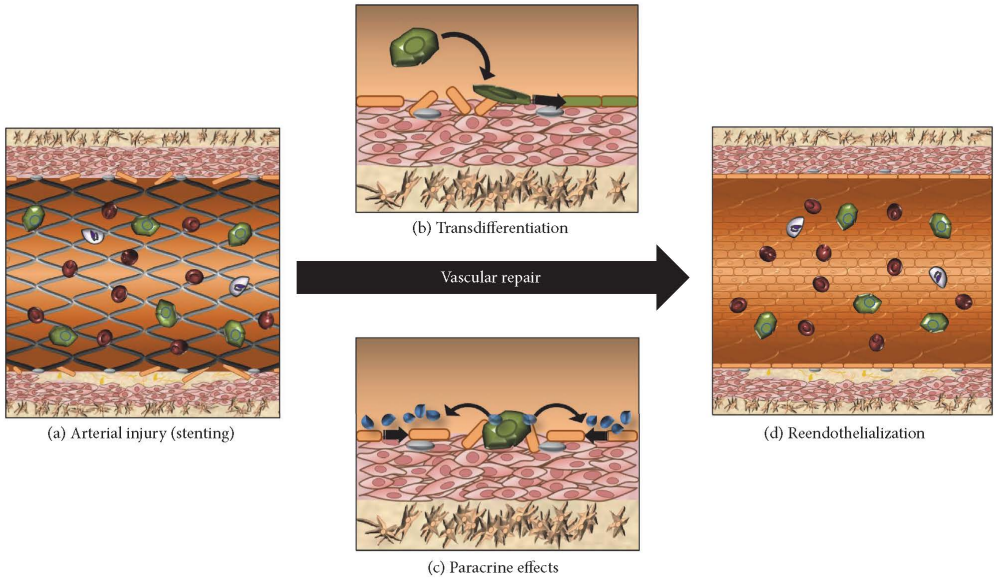


FIGURE 1: Mechanisms of EPC-mediated vascular repair: (a) coronary stenting resulting in arterial injury. Placement of coronary stent to treat obstructive coronary artery disease (CAD) with plaque being pushed into vessel wall and denudation of endothelial cells (ECs) causing vascular injury. Circulating EPCs then home to the site of vascular injury and mediate repair via either transdifferentiation or paracrine effects. (b) Transdifferentiation of EPCs: EPC transdifferentiates into ECs (black arrows) to repair denuded endothelium. (c) Paracrine effects of EPCs: EPCs secrete numerous chemokines (blue) which stimulate surrounding cells to proliferate and migrate towards site of injury to regenerate the EC layer (black arrows). (d) Reendothelialization: endothelial layer is restored with coronary stent underlying regenerated EC layer. Adventitia (brown), media (pink), endothelial cells (orange), EPCs (green), erythrocytes (red), and leukocytes (white).

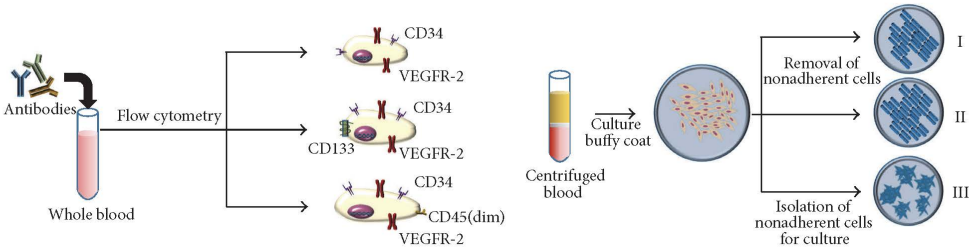


FIGURE 2: Definition of endothelial progenitor cells (EPCs). (a) Flow cytometry: analysis of whole blood samples by flow cytometry utilizing various combinations of antibodies tagged with fluorophores raised against surface antigens such as CD34+/VEGFR-2+, CD34+/VEGFR-2+/CD133+, or CD34+/VEGFR-2+/CD45(dim) to isolate populations. (b) Culture assay: obtained via Ficoll-based centrifugation of whole blood to isolate and culture the buffy-coat layer composed of peripheral blood mononuclear cells (PBMCs). I and II use adherent cells after removal of nonadherent cells. I, EPC culture on fibronectin plate; II, culturing these cells on collagen plate forming endothelial colony forming cells; III, isolation of nonadherent cells for culture to generate colony forming units (CFUs).

2.1.2. Identification by Culture-Based Techniques. EPCs can also be isolated from peripheral blood by plating these cells in various cell culture assays and assessing their colony forming ability (Figure 2). Asahara et al. first identified these CD34+ EPCs based on cluster-forming cells [31]. Colony counts were used as a predictor of the number of circulating progenitors while the spindle-shaped cells surrounding each colony were identified as EPCs. In 2003, Hill et al. isolated peripheral blood mononuclear cells (PBMCs) from venous blood by Ficoll gradient centrifugation. To minimize contamination by mature ECs, PBMCs were plated for 48 hours onto a fibronectin plate and then the nonadherent cells were replated onto a fibronectin-coated plate to quantify EPC-derived colonies. Colonies were then characterized by

Table 1: Evolution of cell-surface markers for endothelial progenitor cell isolation (2014–2016).

		CD34	VEGFR-2/KDR	CD133	CD31	CD45
2014	Liao et al. [51]	Yes	Yes			CD45(dim)
2014	Chi et al. [52]	Yes		Yes		
2015	Shim et al. [53]	Yes			Yes	CD45(dim)
2015	Martí-Fàbregas et al. [54]	Yes	Yes	Yes		
2015	Wang et al. [55]	Yes		Yes		
2015	Tam et al. [56]	Yes	Yes			
2015	Sandra et al. [57]	Yes	Yes	Yes		
2016	Ricottini et al. [58]	Yes	Yes	Yes		CD45(dim)
2016	Lanuti et al. [59]	Yes	Yes	Yes		CD45(dim)
2016	De Ciuceis et al. [60]	Yes	Yes	Yes		
2016	Qin et al. [61]	Yes		Yes		
2016	Niederseer et al., early EPC [62]	Yes				Yes
2016	Niederseer et al., late EPC [62]	Yes	Yes			CD45(dim)
2016	Kung et al. [63]	Yes	Yes	Yes		
2016	Liu et al. [64]	Yes	Yes	Yes		

thin, flat cells which radiated from a centre of clustered cells. The assay was commercialized and these colonies were referred to as colony forming unit-Hill (CFU-Hill). The CFU-Hill assay demonstrated an inverse relationship between the circulating CFU-Hill concentration and Framingham cardiovascular risk score in humans [14]. However, later work called into question the reliability of these units for quantifying EPCs and instead noted they may actually represent cells of macrophage/monocyte and/or T-cell origin [45]. Nonetheless, while CFU-based definitions of EPCs may remain imprecise, they have been shown to serve as a marker of clinical endpoints [14].

Identifying the relationship between these two different isolation methods for EPCs is vital to ensuring a uniform definition of EPCs. One study examined the relationship of both methods and revealed that CFU numbers did not correlate to numbers of either CD34+/KDR+ or CD34+/KDR+/CD133+ cells [46]. In 2007, $CD34^+/VEGFR2^+/CD133^+$ cells were isolated using a cell sorter and assayed using two different endothelial culture systems and hematopoietic colony assays. No endothelial colonies formed and the isolated cells expressed the hematopoietic cell marker, CD45 [44]. One possible explanation for this is that sorted cells may lose critical signaling molecules in the process, which impact the ultimate endothelial phenotype observed in other trials, though this phenomenon is poorly understood. In addition, surface markers used to define EPCs are also found in myeloid-monocytic cells, which may result in isolation of impure EPC populations [47]. Hence, the ideal methodology for isolating and defining an EPC remains the subject of debate, hampering therapeutic advancements.

2.2. Sources of EPCs

2.2.1. Bone Marrow. Asahara et al. described the first EPCs as originating in the bone marrow [31]. The bone marrow cells used expressed LacZ (β-galactosidase), which was transcriptionally regulated by an endothelial-specific promoter (Tie2 or Flk-1). The group identified LacZ-positive cells in the developing neovasculature in all the models [41]. Since then, several other animal models of ischemia have supported the role of bone marrow-derived mononuclear cells in vascular development [48, 49]. Moreover, several clinical trials have employed bone marrow-derived progenitor cells following revascularization for acute myocardial infarction with varying levels of success in terms of mortality and ventricular recovery [50].

2.2.2. Kidney. Renal progenitor cells (RPCs) were isolated from normal adult human kidney cortical parenchyma with cell-surface markers CD133+ and PAX-2+ (embryonic renal marker). These RPCs were capable of self-replication and could be differentiated in vitro into epithelial or endothelial cells [65]. "Nephrospheres" formed by these cells in culture form the basis of a functional assay used for stem cell isolation. One group utilized the PKH26 tracer to identify CD133+/CD24− cells that differentiated into cells with epithelial, endothelial, or podocytic features [66]. It is thought that these resident RPCs may play a role in renal repair in adults though there is limited data to support this [67, 68].

2.2.3. Blood Vessel Wall. Most models of EPC-mediated vascular repair invoke the homing capability of bone marrow-derived EPCs that are circulating in the blood. It is believed that human aortic endothelial cells (HAECs) or human umbilical vein endothelial cells (HUVECs) from vessel walls are terminally differentiated into mature ECs [69]. However, when HAECs were isolated to examine the hierarchy of ECs based on their proliferative capacity, they were able to be passaged in vitro for >40 population doublings using a single-cell deposition assay, thereby suggesting the existence of resident high proliferative potential endothelial colony forming cells (HPP-ECFCs) line, which may assist in vascular repair [69]. This finding suggested that angiogenesis may in fact be mediated by EPCs [70]. However, increasing evidence

suggests that resident ECs actually repair the damaged endothelium, challenging the previous premise that this ability was exclusive to circulating EPCs. Hagensen et al. compared the contribution of circulating and resident cells to vascular repair by transplanting wire-injured carotid artery segments from wild-type mice into Tie2-GFP mice, which expressed GFP in ECs. This model found that the vascular endothelium that formed in response to injury likely arose via migration of surrounding ECs rather than from circulating progenitor cells [71]. Further work is ongoing to identify and characterize the regenerative resident cells within arterial systems.

Recently, in addition to circulating progenitor cells, the existence of resident vascular progenitor cells within the adventitia of blood vessels has been demonstrated. ApoE$^{-/-}$ mice were used to identify adventitial progenitor cells stained by Sca-1, CD34, and Flk-1 [72]. Indeed, Sca-1+ cells were able to differentiate into both ECs and smooth muscle cells (SMCs), thereby potentially contributing to both reendothelialization and/or NI formation, respectively [73]. In 2007, Pasquinelli et al. identified two separate progenitor cell populations between the media and adventitia in human femoral arteries and thoracic aortas [74]. They isolated CD34+ and c-kit+ cells that acquired EC properties when cultured in the presence of VEGF. In 2010, Campagnolo et al. isolated CD34+/CD31− cells from human saphenous veins in CABG patients. These progenitor cells could differentiate into adipocytes, pericytes, and SMCs, stimulated angiogenesis, and improved blood flow when injected into ischemic hind limbs of mice [75].

2.2.4. Adipose Tissue. Adipose tissue was identified as a source of EPCs when Planat-Benard et al. isolated human adipocytes and dedifferentiated them into cells capable of then differentiating into adipocytes or ECs under appropriate conditions [76]. Isolating EPCs from peripheral blood is time consuming whereas adipose tissue may represent an alternative source from which to isolate EPCs. These adipose tissue-derived EPCs (ADEPCs) were capable of enhancing HUVEC capillary-like tube formation on Matrigel [77] and participated in neovascularization when transplanted into rat models of traumatic brain injury [78]. Recent work to characterize the surface markers of ADEPCs has demonstrated the presence of many described EPC markers including CD34, Stro-1, VEGFR-2 (KDR), eNOS, and CD31 [77].

2.2.5. Spleen. Several studies have confirmed an important role for the spleen in EPC mobilization and transplantation [79]. Most hematopoietic stem cells (HSCs) migrate to sinusoids in the bone marrow and spleen and communicate with the endothelium within them, an interaction that seems to be important for their maintenance [80]. Transplantation of spleen-derived mononuclear cells restored endothelium-dependent vasodilation in atherosclerotic ApoE mice, suggesting a splenic source of EPCs for vascular repair [81]. Also, EPCs isolated from spleen homogenate improved reendothelialization and reduced neointima (NI) formation in a model of carotid artery endothelial injury [82].

2.3. EPC-Mediated Vascular Repair

2.3.1. Mobilization. The predominance of evidence supports bone marrow-derived EPCs as the most likely source for arterial repair and accordingly we will focus on their mobilization, reviewing various chemokines responsible for guiding EPCs to sites of vascular injury. VEGF, an effective mobilizer and activator of angiogenesis, is believed to induce proliferation, differentiation, and chemotaxis of ECs following vessel injury [83]. Accordingly, augmenting VEGF levels via an adenovirus that expresses VEGF promoted recruitment of EPCs to sites of injury and promoted neovascularization in mice models. This effect is potentially mediated by matrix metallopeptidase-9 (MMP-9) which is converted to the soluble survival factor sKitL, thereby enhancing VEGFR2+ progenitor cells and facilitating transport from osteoblast-rich locations to vascular areas, promoting movement into the circulation [84]. Interestingly, nitric oxide (NO) appears to play a major role in the expression of MMP-9 in its inactive and active form. Studies of endothelial nitric oxide synthase 3 knockout mice (NOS3 KO) have shown reduced expression and activity of MMP-9 even after stimulation with VEGF. These same mice demonstrated reduced neovascularization with induced ischemia and reduced EPC mobilization to ischemic areas [85]. In contrast, AVE9488 (eNOS upregulator) increased NO expression in EPCs and enhanced their migratory capacity [86]. Thus, it appears that NO and MMP-9 play a critical role in facilitating VEGF-mediated angiogenesis.

Secondarily, SDF-1 is a chemokine of growing interest, a constitutively expressed protein that is upregulated by inflammatory mediators, extracellular matrix changes, mechanical forces, and hypoxia [87]. Platelets secrete SDF-1 upon being activated at sites of injury, which may provide the local signal needed to recruit EPCs to the site of injury. In fact, SDF-1 seems to only induce neovascularization in the presence of ischemic injury [88]. In NOS3 KO mice, SDF-1-associated neovascularization and EPC mobilization were silenced, suggesting that VEGF/eNOS signaling pathways likely play important roles in this vascular homeostasis [89]. Additional factors with similar function to SDF-1 include estrogen, statins, and erythropoietin. These factors improve EPC mobilization and neovascularization and inhibit neointima hyperplasia but are similarly silenced in NOS3 KO mice [90–93].

2.3.2. Homing. Understanding the mechanism by which EPCs home to sites of vascular injury is critical to harnessing their therapeutic potential. This process is thought to be similar to the rolling and adhesion behaviour exhibited by leukocytes in the setting of inflammation. EPC homing involves an interaction between EPC surface molecules and associated ligands expressed on dying ECs. P- and E-selectin are believed to play critical roles in this process. Foubert et al. have shed light on this process through their studies on the erythropoietin-producing human hepatocellular carcinoma (Eph) receptor and its associated ephrin ligands, which are regulators of vascular development. The group has shown that Eph4B activation by ephrin-B2-Fc chimeric protein increases the angiogenic potential of human EPCs in a hind limb

ischemia mouse model and that this response is blunted with EphB4 siRNA treatment. Eph4B appears to elevate P-selectin glycoprotein ligand-1 expression and EPC adhesion, an effect that can be abrogated with utilizing neutralizing antibodies to E- and P-selectin [94]. β2-Integrins on the surface of EPCs mediate migration and adhesion of EPCs to the damaged endothelium as well as neovascularization [95]. High motility group box 1 (HMGB1) has been shown to activate β1- and β2-integrins on the surface of EPCs by binding to HMGB1 receptors RAGE (receptor for advanced glycation endproducts) and TLR2 (toll-like receptor 2). Interestingly, HMGB1 is released into the extracellular space upon necrosis, but not apoptosis, and was associated with improved homing and adhesion of EPCs to the site of injury [96].

Next, intercellular adhesion molecule-1 (ICAM-1) upregulation in ischemia has been noted to increase EPC recruitment to ischemic limbs [97]. As a hypoxia-responsive gene, integrin-linked kinase (ILK) regulates ICAM-1 expression. Its overexpression is thus associated with increased expression of ICAM-1 as well as SDF-1 in ECs, two key molecules involved in the recruitment of EPCs during vasculogenesis [98]. The synergy between EPC surface proteins and endothelial and/or extracellular ligands is therefore essential for EPC homing to sites of injury (Figure 1).

Apart from EPC migration to sites of vascular injury, EPC invasion of injured tissue is critical for organ repair and function. Gene expression profiling of EPCs and mature ECs has identified the protease Cathepsin L (CathL) as being highly expressed. CathL was shown in vitro to be crucial for matrix degradation and EPC invasion. A CathL-deficient hind limb ischemia mouse model characteristically shows poor limb recovery. As well, when CathL-deficient progenitor cells are infused, impaired homing to sites of injury and impaired neovascularization are observed [99]. EPCs lacking MMP-2 similarly show reduced invasiveness and proliferative capabilities [100]. Hence, numerous molecules have been implicated in the homing of EPCs via a rolling and adhesion model, with additional protease-mediated invasion as required.

2.3.3. Paracrine Effects. It is unlikely that EPC transdifferentiation into mature ECs represents a meaningful reparative mechanism. Rather, an increasing number of studies have shown that these circulating cells may facilitate arterial repair through paracrine influence on neighbouring cells (Figure 1). Many of the cells previously identified as EPCs have more recently been shown to derive from a monocytic lineage, suggesting that they may contribute to vascular repair by releasing paracrine factors such as VEGF [101]. For example, treatment of HUVECs and coronary artery endothelial cells (CAECs) with cultured media (CM) from EPCs or mature ECs demonstrated that EPC-derived CM has higher levels of IL-8 and it demonstrated a stimulatory effect on HUVEC and CAEC proliferation [102].

Paracrine signaling appears to be an important aspect in both early and late EPCs. Early EPCs are derived from a monocytic cell lineage (CD14+) to form spindle-shaped cells after 7 days of culture. In contrast, late EPCs are derived from a CD34+ lineage after 14–28 days in culture and resemble ECs [39]. Early EPCs have a modest contribution to the direct incorporation into the endothelium relative to late EPCs [103] but secrete proangiogenic stimulatory cytokines such as VEGF, fibroblast growth factor (FGF), IL-8, placental growth factor, SDF-1, and platelet derived growth factor (PDGF) [104, 105]. Additionally, the manipulation of the VEGF-Akt pathway could stimulate cell survival and proliferation [106]. These signals are critical as they contribute to endothelial regeneration by recruiting resident endothelial and cardiac progenitor cells to improve endothelial regeneration. Late EPCs have a direct role in neovascularization but a reduced role in paracrine signaling compared to early EPCs. However, they still have been shown to secrete proangiogenic factors including VEGF, IL-8, and PDGF [107]. They suggest that further subsets of EPCs could be defined based on their secretion profile, while modulation of their chemokine profile may help improve their inflammatory profile [108].

More recently, EC-derived microparticles (EMPs) or microvesicles (MV) have been identified as vehicles for different paracrine modes of action. EMPs are complex vesicles shed from activated or apoptotic ECs which play a major role in endothelial function and angiogenesis. EMPs are highly functional molecules with several surface molecules and contain DNA, RNA, or microRNA [109]. These EMPs are taken up by target cells, such as mature ECs, to stimulate endothelial regeneration [47]. Injection of EMPs has been associated with attenuated kidney injury in rats by enhancing proliferation and decreasing apoptosis. This effect was reduced after treating EMPs with RNase or miRNA depletion, implicating a potential function of RNA within EMP for vascular repair [110]. Others have shown that treatment with EMPs accelerates endothelial repair after carotid artery injury in mouse models and that miRNA-126 within EMPs plays a critical role in regulating EC mobilization, proliferation, and regeneration [111]. As we continue to decipher the underlying biology of progenitor cells, the impact of paracrine signaling for vascular repair appears to grow evermore important, presenting both obstacles but also more potential therapeutic targets.

3. Therapeutic Potential

Preclinical trials involving EPCs have shown promising results, with EPCs demonstrating the ability to specifically incorporate into the site of injury and mitigate neointima formation [112]. Our group isolated cultured angiogenic cells from patients with CAD and showed that improving CACs could improve reendothelialization [42]. Other substances, including estrogen, G-CSF, and leptin have been demonstrated to mobilize EPCs and enhance arterial repair after vascular injury in animal models [113–115]. Accordingly, endothelial dysfunction is thought to portend cardiovascular disease. Thus, circulating EPC levels may serve as a potential biomarker of endothelial function and integrity, while augmenting EPC levels may represent a viable therapeutic target. Understanding and augmenting endogenous EPCs have attracted considerable attention thus far, so does the transplantation of EPCs directly into sites of injuries. Indeed, these two therapeutic themes represent the majority of applications of EPCs in a therapeutic role to date. However, clinical trials augmenting EPC levels and activity have great

variation in EPC isolation techniques and definitions, likely contributing to conflicting results and hindering the advancement of EPCs in the therapeutic realm.

3.1. Augmenting Endogenous EPCs

3.1.1. Antihypertensive Therapy. Some common antihypertensive (i.e., blood pressure lowering) agents include angiotensin converting enzyme inhibitors (ACEi), angiotensin II receptor blockers (ARB), and calcium channel blockers (CCB). All of these agents have been extensively studied and yielded an improvement in EPC number and function by differing mechanisms of action. ARBs have improved numbers and function of EPCs by inhibiting oxidative stress [116], while ACEi yielded augmented EPC number and function when given to patients with stable CAD [117]. ACEi have also demonstrated stimulation of nitric oxide activity and diminished oxidative stress in human cells [118]. CCBs such as nifedipine improved EPC function and provided a greater resistance to oxidative stress and apoptosis [119]. Hence, considerable evidence supports the role for antihypertensive agents in improving EPC number and function.

3.1.2. Statin Therapy. Statins were originally developed to modify lipid profiles in patients by reducing LDL and triglyceride levels to improve cardiovascular outcomes. However, clinical studies involving statin therapy in patients with CAD showed higher levels and mobilization of EPCs [120, 121]. These studies showed the initial promise of potentially enhancing endogenous EPCs to improve clinical outcomes. The mechanism behind statin's effect on EPCs is a dose-dependent augmentation of Akt phosphorylation within minutes, which yields an increase in mobilization, migration, proliferation, and survival of EPCs [120]. Elevated EPC levels have also been observed with statin therapy in patients with CAD, acute myocardial infarction, and post-CABG [122–124]. From a vascular repair perspective, patients who develop ISR have also been shown to have functionally impaired EPCs [16, 17]. In a previous review, we demonstrated that statin therapy resulted in a significant rise in circulating EPCs over control, with a median increase of 70.9% (range from 25.8% to 223.5%) [43]. The considerable variance in EPC augmentation following statin therapy most likely reflects differences in the definition of EPCs employed as well as varying isolation techniques.

3.1.3. GSK-3β. Glycogen synthase kinase (GSK-3β) is a serine/threonine kinase that phosphorylates β-catenin to negatively regulate the Wnt signaling pathway [125]. The Wnt signaling pathway plays an essential role in mobilization of EPCs and enhancement of neovascularization [126]. This pathway produces multiple secreted glycoproteins which regulate many cell processes, including hematopoiesis and stem cell function. Our group showed for the first time in an in vitro experiment that the inhibition of GSK-3β was associated with increase in EPC levels and reduction of EPC apoptosis. In vivo, we observed that GSK-3β inhibition resulted in an improvement in reendothelialization and reduction of neointima formation following injury [42].

Patients with diabetes mellitus (DM) have reduced EPC levels and increased rates of apoptosis. In addition, diabetics have shown higher level of GSK-3β activity that resulted in higher levels of phosphorylated β-catenin. DM-EPCs are associated with reduced mobilization, migration, and homing to sites of injury [127]. Our group has shown that treatment with GSK-3β inhibitors reduced apoptosis, increased VEGF production, and enhanced EPC invasive capacity in vitro. Proteomics analysis of DM-EPC versus normal EPC revealed 37 uniquely regulated proteins. Cathepsin B was identified as the protein that mediates enhanced invasive capacity of EPCs after GSK-3β inhibition [128]. Although the direct mechanism of action remains unclear, other studies appear to support our findings. Activation of Wnt/β-catenin pathway during human mesenchymal stem cell differentiation was associated with upregulation of Cathepsin B [129]. Although more studies are required on the application of GSK-3β inhibition to enhance EPC function, it appears to be a promising avenue for future therapeutic development.

3.1.4. Stents. As discussed, coronary stents have markedly advanced from simple metal scaffolds to sophisticated drug-delivery systems to facilitate arterial healing [5]. Drug-eluting stents (DESs) routinely used for PCI are coated with antiproliferative and anti-inflammatory agents, with first-generation DESs utilizing sirolimus and paclitaxel. Paclitaxel was originally developed as a treatment for ovarian cancer, preventing cellular proliferation via inhibition of microtubule regulation during mitosis [130]. Subsequent work then demonstrated its affinity for inhibiting smooth muscle cell and neointima formation, leading to its development for DESs [131]. Sirolimus (rapamycin) was originally developed as an antifungal agent, but its use was limited due to its immunosuppressive properties. However, it was also noted to suppress NI formation lending itself well to DES implementation. It enacts its effects via blocking G_1 to S phase progression in cell cycle via inhibition of mammalian target of rapamycin (mTOR) kinase [132]. As the role of EPCs in vascular healing became apparent, novel technologies then focused on discovering means of increasing EPC recruitment and proliferation specifically at the site of stent deployment and subsequent endothelial injury [133].

The first stent to harness the healing capacity of EPCs was the Genous monoclonal anti-human CD34 coated stent [134]. This EPC-capture stent consisted of a stainless steel scaffold covered in a covalently coupled polysaccharide polymer with CD34 antibodies. The Healing-FIM trial compared EPC-capture stent versus bare-metal stents, demonstrating similar NI hyperplasia between the two cohorts and suggesting that the EPC-capture stent failed to inhibit NI hyperplasia [134]. While initially a promising concept, subsequent randomized trials have actually revealed increased ISR with the Genous stent compared to the paclitaxel-eluting stent, paradoxically suggesting increased NI hyperplasia with this technology [135]. These disappointing results are felt to be related to three factors: (i) the stent scaffold itself was an early generation device (thicker struts without any antiproliferative medication), (ii) CD34 is a surface antigen common to various progenitors and may result in nonspecific binding

to the stent thereby promoting NI hyperplasia, and (iii) patients with CAD are known to have reduced number and functionality of EPCs [5, 15]. Moreover, the HEALING II registry reported that patients with normal levels of CD34+KDR+ EPC titers had lower rates of ISR compared to patients with reduced levels of EPCs – supporting the notion that augmentation of endogenous EPCs may be key to this success [136]. Hence, attempting to harness rare and dysfunctional cells is likely of little benefit. Accordingly, future trials tried to address the state of the endogenous EPCs available by combining statin therapy with Genous stenting to improve reendothelialization [137]. In a recent 5-year follow-up study of patients treated with Genous stent target lesion revascularization, stent thrombosis, and ISR were stabilized within 12–24 months and up to 5 years [138]. Further data is needed to better understand the role of EPC-capture stent technology in conventional PCI.

To further refine this technology, the EPC-capture stent has been combined with a paclitaxel-coated balloon with intent of reducing the noted NI proliferation and ISR that plagued first-generation Genous stents given its antiproliferative properties. The Perfect Stent study studied 120 patients with CAD who were treated with EPC-capture stent followed by paclitaxel-eluting balloon after dilatation. This approach was successful in reducing restenosis [139] and may well represent the future direction of this technology. Similarly, the Combo stent (OrbusNeich Medical, Fort Lauderdale, USA) is composed of EPC capturing technology combined with a sirolimus-eluting stent to minimize NI formation. The total concentration of sirolimus is half of what is found in a standard DES, but it is released in the same fashion. Preclinical trials in porcine models showed reduced NI thickness in the Combo stent compared to the standard sirolimus-eluting stent, low-dose Combo stent, and everolimus (sirolimus-analog) eluting stent [140]. In 2013, Haude et al. published the prospective, multicenter, randomized evaluation of an abluminal sirolimus coated bioengineered stent (REMEDEE) trial, comparing the Combo stent with the paclitaxel-eluting Taxus Liberte stent and demonstrate that the Combo stent was superior to the paclitaxel stent at 9-month angiographic follow-up because of in-stent late lumen loss with values of 0.39 ± 0.45 mm and 0.44 ± 0.56 mm, respectively [141]. This demonstrated the initial safety and efficacy of Combo stents in comparison to first-generation DESs, but further data is still required to establish this technology. While augmenting and maximizing endogenous EPCs show some promise, an alternative approach involves the direct administration of exogenous EPCs.

3.2. Transplantation of Exogenous EPCs. Stem cell therapies have been developed as new treatment regimen for patients suffering from acute myocardial infarctions since the early 2000s. The first of these trials involved the injection of bone marrow-derived mononuclear cells (BMMNC) in 10 patients at the site of infarct following balloon dilatation [142]. Since then, numerous trials injecting BMMNC have been conducted following revascularization by both PCI and CABG to assess myocardial recovery [143–145]. This success has led to large randomized controlled trials of progenitor cell transplantation worldwide [146–148], including myocardial infarction [149].

The procedure involves harvesting bone marrow under general anesthesia from the bony pelvis. CD34+ or CD133+ hematopoietic progenitor cells (similar surface markers as the EPCs defined earlier) are isolated from the BMMNC and then cultured for 2–4 weeks to obtain a sufficient yield of progenitor cells. These cells are then injected directly into the patient's heart at the site of injury during PCI. The timing of stem cell injection varies in studies from first 24–48 hours to four weeks after PCI. Also, the dose of cells that were administered varied considerably within these large trials anywhere from 10^6 to 10^{10} cells [146–149]. Overall, major trials showed no significant mortality difference between those who received stem cell transplant and those who did not receive cells in the short term. In addition, a Cochrane review on stem cell treatment for acute myocardial infarction revealed no significant reduction in cardiovascular mortality or major adverse cardiovascular events after cell therapy in short- and long-term follow-up [50]. While certainly intriguing and promising, this technology is still in its infancy, and considerable advancements are needed for this to establish itself as a legitimate therapeutic option.

4. Conclusion

Our understanding of the complex environment that is the vessel wall remains incomplete. Certainly, the discovery of EPCs holds great potential for both the monitoring and therapy of vascular disease, particularly in repairing the endothelial injury incurred by modern interventions. Be it by augmentation and exploitation of endogenous cells or direct transplantation of cells to areas of need, both approaches show promise. However, despite considerable efforts over the past few decades, our understanding of the definition, signaling, and differentiation of these enigmatic progenitor cells remains imperfect, hampering further developments. Only once these fundamental components are clarified will progenitor cells be able to advance and emerge as a legitimate therapeutic option.

Disclosure

Trevor Simard and Richard G. Jung shared first authorship.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

References

[1] D. G. Manuel, M. Leung, K. Nguyen, P. Tanuseputro, and H. Johansen, "Burden of cardiovasc disease in Canada," *Canadian Journal of Cardiology*, vol. 19, no. 9, pp. 997–1004, 2003.

[2] E. M. Antman, D. T. Anbe, P. W. Armstrong et al., "ACC/AHA guidelines for the management of patients with ST-elevation myocardial infarction–executive summary. A report of the American College of Cardiology/American Heart Association

Task Force on Practice Guidelines (Writing Committee to revise the 1999 guidelines for the management of patients with acute myocardial infarction)," *Journal of the American College of Cardiology*, vol. 44, no. 3, pp. 671–719, 2004.

[3] W. E. Boden, R. A. O'Rourke, K. K. Teo et al., "Optimal medical therapy with or without PCI for stable coronary disease," *New England Journal of Medicine*, vol. 356, no. 15, pp. 1503–1516, 2007.

[4] M. Froeschl, S. Olsen, X. Ma, and E. R. O'Brien, "Current understanding of in-stent restenosis and the potential benefit of drug eluting stents," *Current Drug Targets—Cardiovascular and Haematological Disorders*, vol. 4, no. 1, pp. 103–117, 2004.

[5] T. Simard, B. Hibbert, F. D. Ramirez, M. Froeschl, Y.-X. Chen, and E. R. O'Brien, "The evolution of coronary stents: a brief review," *Canadian Journal of Cardiology*, vol. 30, no. 1, pp. 35–45, 2014.

[6] K. H. Bønaa, J. Mannsverk, R. Wiseth et al., "Drug-eluting or bare-metal stents for coronary artery disease," *New England Journal of Medicine*, vol. 375, no. 13, pp. 1242–1252, 2016.

[7] L. Mauri, W.-H. Hsieh, J. M. Massaro, K. K. L. Ho, R. D'Agostino, and D. E. Cutlip, "Stent thrombosis in randomized clinical trials of drug-eluting stents," *New England Journal of Medicine*, vol. 356, no. 10, pp. 1020–1029, 2007.

[8] A. Farb, G. Sangiorgi, A. J. Carter et al., "Pathology of acute and chronic coronary stenting in humans," *Circulation*, vol. 99, no. 1, pp. 44–52, 1999.

[9] V. J. Dzau, M. Gnecchi, A. S. Pachori, F. Morello, and L. G. Melo, "Therapeutic potential of endothelial progenitor cells in cardiovascular diseases," *Hypertension*, vol. 46, no. 1, pp. 7–18, 2005.

[10] K.-H. Mak, G. Belli, S. G. Ellis, and D. J. Moliterno, "Subacute stent thrombosis: evolving issues and current concepts," *Journal of the American College of Cardiology*, vol. 27, no. 2, pp. 494–503, 1996.

[11] R. Muniyappa and J. R. Sowers, "Glycogen synthase kinase-3β and cathepsin B in diabetic endothelial progenitor cell dysfunction: an old player finds a new partner," *Diabetes*, vol. 63, no. 4, pp. 1194–1197, 2014.

[12] J. N. Wilcox, E.-I. Okamoto, K.-I. Nakahara, and J. Vinten-Johansen, "Perivascular responses after angioplasty which may contribute to postangioplasty restenosis," *Annals of the New York Academy of Sciences*, vol. 947, no. 1, pp. 68–92, 2001.

[13] T. Asahara, T. Takahashi, H. Masuda et al., "VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells," *EMBO Journal*, vol. 18, no. 14, pp. 3964–3972, 1999.

[14] J. M. Hill, G. Zalos, J. P. J. Halcox et al., "Circulating endothelial progenitor cells, vascular function, and cardiovascular risk," *New England Journal of Medicine*, vol. 348, no. 7, pp. 593–600, 2003.

[15] N. Werner, S. Kosiol, T. Schiegl et al., "Circulating endothelial progenitor cells and cardiovascular outcomes," *New England Journal of Medicine*, vol. 353, no. 10, pp. 999–1007, 2005.

[16] J. George, I. Herz, E. Goldstein et al., "Number and adhesive properties of circulating endothelial progenitor cells in patients with in-stent restenosis," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 23, no. 12, pp. e57–e60, 2003.

[17] B. Hibbert, Y.-X. Chen, and E. R. O'Brien, "c-kit-immunopositive vascular progenitor cells populate human coronary in-stent restenosis but not primary atherosclerotic lesions," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 287, no. 2, pp. H518–H524, 2004.

[18] V. Schächinger, S. Erbs, A. Elsässer et al., "Improved clinical outcome after intracoronary administration of bone-marrow-derived progenitor cells in acute myocardial infarction: final 1-year results of the REPAIR-AMI trial," *European Heart Journal*, vol. 27, no. 23, pp. 2775–2783, 2006.

[19] N. Werner, S. Junk, U. Laufs et al., "Intravenous transfusion of endothelial progenitor cells reduces neointima formation after vascular injury," *Circulation research*, vol. 93, no. 2, pp. e17–e24, 2003.

[20] J. George, E. Goldstein, S. Abashidze et al., "Circulating endothelial progenitor cells in patients with unstable angina: association with systemic inflammation," *European Heart Journal*, vol. 25, no. 12, pp. 1003–1008, 2004.

[21] M. Massa, V. Rosti, M. Ferrario et al., "Increased circulating hematopoietic and endothelial progenitor cells in the early phase of acute myocardial infarction," *Blood*, vol. 105, no. 1, pp. 199–206, 2005.

[22] G. J. Padfield, D. E. Newby, and N. L. Mills, "Understanding the role of endothelial progenitor cells in percutaneous coronary intervention," *Journal of the American College of Cardiology*, vol. 55, no. 15, pp. 1553–1565, 2010.

[23] H. W. Florey, S. J. Greer, J. C. Poole, and N. T. Werthessen, "The pseudointima lining fabric grafts of the aorta," *British Journal of Experimental Pathology*, vol. 42, pp. 236–246, 1961.

[24] G. L. Jordan, M. M. Stump, M. E. de Bakey, and B. Halpert, "Endothelial lining of dacron prostheses of porcine thoracic aortas," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 110, no. 2, pp. 340–343, 1962.

[25] M. M. Stump, G. L. Jordan, M. E. De Bakey, and B. Halpert, "The endothelial lining of homografts and dacron prostheses in the canine aorta," *The American Journal of Pathology*, vol. 40, pp. 487–491, 1962.

[26] M. M. Stump, G. L. Jordan Jr., M. E. Debakey, and B. Halpert, "Endothelium grown from circulating blood on isolated intravascular," *The American journal of pathology*, vol. 43, pp. 361–367, 1963.

[27] W. Feigl, M. Susani, W. Ulrich, M. Matejka, U. Losert, and H. Sinzinger, "Organisation of experimental thrombosis by blood cells—evidence of the transformation of mononuclear cells into myofibroblasts and endothelial cells," *Virchows Archiv A Pathological Anatomy and Histopathology*, vol. 406, no. 2, pp. 133–148, 1985.

[28] G. Pasquinelli, P. Preda, T. Curti, M. D'Addato, and R. Laschi, "Endothelialization of a new Dacron graft in an experimental model: light microscopy, electron microscopy and immunocytochemistry," *Scanning Microscopy*, vol. 1, no. 3, pp. 1327–1338, 1987.

[29] J. H. Campbell, J. L. Efendy, and G. R. Campbell, "Novel vascular graft grown within recipient's own peritoneal cavity," *Circulation Research*, vol. 85, no. 12, pp. 1173–1178, 1999.

[30] B. Hibbert, T. Simard, and E. R. O'Brien, "Endothelial progenitors and repair of cardiovascular disease," in *Regenerative Therapy Using Blood-Derived Stem Cells*, D. S. Allan and D. Strunk, Eds., pp. 97–107, Springer, 2012.

[31] T. Asahara, T. Murohara, A. Sullivan et al., "Isolation of putative progenitor endothelial cells for angiogenesis," *Science*, vol. 275, no. 5302, pp. 964–967, 1997.

[32] B. Hibbert, X. Ma, A. Pourdjabbar et al., "Pre-procedural Atorvastatin mobilizes endothelial progenitor cells: clues to the salutary effects of statins on healing of stented human arteries," *PLOS ONE*, vol. 6, no. 1, Article ID e16413, 2011.

[33] S. Janssens, C. Dubois, J. Bogaert et al., "Autologous bone marrow-derived stem-cell transfer in patients with ST-segment elevation myocardial infarction: double-blind, randomised controlled trial," *The Lancet*, vol. 367, no. 9505, pp. 113–121, 2006.

[34] X. Ma, B. Hibbert, B. Dhaliwal et al., "Delayed re-endothelialization with rapamycin-coated stents is rescued by the addition of a glycogen synthase kinase-3 inhibitor," *Cardiovascular Research*, vol. 86, no. 2, pp. 338–345, 2010.

[35] T. Takahashi, C. Kalka, H. Masuda et al., "Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization," *Nature Medicine*, vol. 5, no. 4, pp. 434–438, 1999.

[36] K. A. Jackson, S. M. Majka, H. Wang et al., "Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells," *Journal of Clinical Investigation*, vol. 107, no. 11, pp. 1395–1402, 2001.

[37] T. Asahara, H. Masuda, T. Takahashi et al., "Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization," *Circulation Research*, vol. 85, no. 3, pp. 221–228, 1999.

[38] D. Lyden, K. Hattori, S. Dias et al., "Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth," *Nature Medicine*, vol. 7, no. 11, pp. 1194–1201, 2001.

[39] S. Xu, J. Zhu, L. Yu, and G. Fu, "Endothelial progenitor cells: current development of their paracrine factors in cardiovascular therapy," *Journal of Cardiovascular Pharmacology*, vol. 59, no. 4, pp. 387–396, 2012.

[40] F. Timmermans, J. Plum, M. C. Yöder, D. A. Ingram, B. Vandekerckhove, and J. Case, "Endothelial progenitor cells: identity defined?" *Journal of Cellular and Molecular Medicine*, vol. 13, no. 1, pp. 87–102, 2009.

[41] A. A. Sirker, Z. M. J. Astroulakis, and J. M. Hill, "Vascular progenitor cells and translational research: the role of endothelial and smooth muscle progenitor cells in endogenous arterial remodelling in the adult," *Clinical Science*, vol. 116, no. 4, pp. 283–299, 2009.

[42] B. Hibbert, X. Ma, A. Pourdjabbar et al., "Inhibition of endothelial progenitor cell glycogen synthase kinase-3β results in attenuated neointima formation and enhanced re-endothelialization after arterial injury," *Cardiovascular Research*, vol. 83, no. 1, pp. 16–23, 2009.

[43] B. Hibbert, T. Simard, F. D. Ramirez et al., "The effect of statins on circulating endothelial progenitor cells in humans: a systematic review," *Journal of Cardiovascular Pharmacology*, vol. 62, no. 5, pp. 491–496, 2013.

[44] J. Case, L. E. Mead, W. K. Bessler et al., "Human CD34+AC133+VEGFR-2+ cells are not endothelial progenitor cells but distinct, primitive hematopoietic progenitors," *Experimental Hematology*, vol. 35, no. 7, pp. 1109–1118, 2007.

[45] J. C. Kovacic and M. Boehm, "Resident vascular progenitor cells: an emerging role for non-terminally differentiated vessel-resident cells in vascular biology," *Stem Cell Research*, vol. 2, no. 1, pp. 2–15, 2009.

[46] J. George, H. Shmilovich, V. Deutsch, H. Miller, G. Keren, and A. Roth, "Comparative analysis of methods for assessment of circulating endothelial progenitor cells," *Tissue Engineering*, vol. 12, no. 2, pp. 331–335, 2006.

[47] M. Zhang, A. B. Malik, and J. Rehman, "Endothelial progenitor cells and vascular repair," *Current Opinion in Hematology*, vol. 21, no. 3, pp. 224–228, 2014.

[48] A. Kawamoto, H.-C. Gwon, H. Iwaguro et al., "Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia," *Circulation*, vol. 103, no. 5, pp. 634–637, 2001.

[49] A. A. Kocher, M. D. Schuster, M. J. Szabolcs et al., "Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function," *Nature Medicine*, vol. 7, no. 4, pp. 430–436, 2001.

[50] S. A. Fisher, H. Zhang, C. Doree, A. Mathur, and E. Martin-Rendon, "Stem cell treatment for acute myocardial infarction," *The Cochrane Database of Systematic Reviews*, no. 9, p. CD006536, 2015.

[51] Y.-F. Liao, Y. Feng, L.-L. Chen, T.-S. Zeng, F. Yu, and L.-J. Hu, "Coronary heart disease risk equivalence in diabetes and arterial diseases characterized by endothelial function and endothelial progenitor cell," *Journal of Diabetes and Its Complications*, vol. 28, no. 2, pp. 214–218, 2014.

[52] J. Chi, X. Hong, Y. Wang, J. Zhao, and W. Yang, "Inverse correlation between circulating endothelial progenitor cells with CD34+CD133+ and the severity of coronary atherosclerosis assessed by syntax score," *American Journal of the Medical Sciences*, vol. 347, no. 6, pp. 457–462, 2014.

[53] Y. Shim, M. H. Nam, S. W. Hyuk, S. Y. Yoon, and J. M. Song, "Concurrent hypermulticolor monitoring of CD31, CD34, CD45 and CD146 endothelial progenitor cell markers for acute myocardial infarction," *Analytica Chimica Acta*, vol. 853, pp. 501–507, 2015.

[54] J. Martí-Fàbregas, R. Delgado-Mederos, J. Crespo et al., "Circulating endothelial progenitor cells and the risk of vascular events after ischemic stroke," *PLOS ONE*, vol. 10, no. 4, Article ID e0124895, 2015.

[55] L. Wang, X. Wang, H. Su et al., "Recombinant human erythropoietin improves the neurofunctional recovery of rats following traumatic brain injury via an increase in circulating endothelial progenitor cells," *Translational Stroke Research*, vol. 6, no. 1, pp. 50–59, 2015.

[56] J. C. W. Tam, C. H. Ko, K. M. Lau et al., "Enumeration and functional investigation of endothelial progenitor cells in neovascularization of diabetic foot ulcer rats with a Chinese 2-herb formula," *Journal of Diabetes*, vol. 7, no. 5, pp. 718–728, 2015.

[57] F. Sandra, Y. H. Oktaviono, M. A. Widodo, Y. Dirgantara, A. Chouw, and D. Sargowo, "Endothelial progenitor cells proliferated via MEK-dependent p42 MAPK signaling pathway," *Molecular and Cellular Biochemistry*, vol. 400, no. 1-2, pp. 201–206, 2015.

[58] E. Ricottini, R. Madonna, D. Grieco et al., "Effect of high-dose atorvastatin reload on the release of endothelial progenitor cells in patients on long-term statin treatment who underwent percutaneous coronary intervention (from the ARMYDA-EPC Study)," *American Journal of Cardiology*, vol. 117, no. 2, pp. 165–171, 2016.

[59] P. Lanuti, G. Rotta, C. Almici et al., "Endothelial progenitor cells, defined by the simultaneous surface expression of VEGFR2 and CD133, are not detectable in healthy peripheral and cord blood," *Cytometry Part A*, vol. 89, no. 3, pp. 259–270, 2016.

[60] C. De Ciuceis, C. Rossini, A. Tincani et al., "Effect of antihypertensive treatment with lercanidipine on endothelial progenitor cells and inflammation in patients with mild to moderate essential hypertension," *Blood Pressure*, vol. 25, no. 6, pp. 337–343, 2016.

[61] G. Qin, Y. Chen, H. Li et al., "Melittin inhibits tumor angiogenesis modulated by endothelial progenitor cells associated with the SDF-1α/CXCR4 signaling pathway in a UMR-106 osteosarcoma xenograft mouse model," *Molecular Medicine Reports*, vol. 14, no. 1, pp. 57–68, 2016.

[62] D. Niederseer, E. Steidle-Kloc, M. Mayr et al., "Effects of a 12-week alpine skiing intervention on endothelial progenitor cells, peripheral arterial tone and endothelial biomarkers in the elderly," *International Journal of Cardiology*, vol. 214, pp. 343–347, 2016.

[63] C.-T. Kung, C.-M. Su, C. T. Chen et al., "Circulating endothelial progenitor cells may predict outcomes in adult patients with severe sepsis in the emergency department," *Clinica Chimica Acta*, vol. 455, pp. 1–6, 2016.

[64] J.-H. Liu, Y. Chen, Z. Zhen et al., "Relation between endothelial progenitor cells and arterial stiffness in patients with psoriasis," *Journal of Dermatology*, vol. 43, no. 8, pp. 888–893, 2016.

[65] B. Bussolati, S. Bruno, C. Grange et al., "Isolation of renal progenitor cells from adult human kidney," *American Journal of Pathology*, vol. 166, no. 2, pp. 545–555, 2005.

[66] S. Bombelli, M. A. Zipeto, B. Torsello et al., "PKHhigh cells within clonal human nephrospheres provide a purified adult renal stem cell population," *Stem Cell Research*, vol. 11, no. 3, pp. 1163–1177, 2013.

[67] B. Bussolati and G. Camussi, "Therapeutic use of human renal progenitor cells for kidney regeneration," *Nature Reviews Nephrology*, vol. 11, no. 12, pp. 695–706, 2015.

[68] M. S. Goligorsky, K. Yasuda, and B. Ratliff, "Dysfunctional endothelial progenitor cells in chronic kidney disease," *Journal of the American Society of Nephrology*, vol. 21, no. 6, pp. 911–919, 2010.

[69] D. A. Ingram, L. E. Mead, D. B. Moore, W. Woodard, A. Fenoglio, and M. C. Yoder, "Vessel wall-derived endothelial cells rapidly proliferate because they contain a complete hierarchy of endothelial progenitor cells," *Blood*, vol. 105, no. 7, pp. 2783–2786, 2005.

[70] J. C. Kovacic, J. Moore, A. Herbert, D. Ma, M. Boehm, and R. M. Graham, "Endothelial progenitor cells, angioblasts, and angiogenesis—old terms reconsidered from a current perspective," *Trends in Cardiovascular Medicine*, vol. 18, no. 2, pp. 45–51, 2008.

[71] M. K. Hagensen, M. K. Raarup, M. B. Mortensen et al., "Circulating endothelial progenitor cells do not contribute to regeneration of endothelium after murine arterial injury," *Cardiovascular Research*, vol. 93, no. 2, pp. 223–231, 2012.

[72] Y. Hu, Z. Zhang, E. Torsney et al., "Abundant progenitor cells in the adventitia contribute to atherosclerosis of vein grafts in ApoE-deficient mice," *Journal of Clinical Investigation*, vol. 113, no. 9, pp. 1258–1265, 2004.

[73] E. Torsney, Y. Hu, and Q. Xu, "Adventitial progenitor cells contribute to arteriosclerosis," *Trends in Cardiovascular Medicine*, vol. 15, no. 2, pp. 64–68, 2005.

[74] G. Pasquinelli, P. L. Tazzari, C. Vaselli et al., "Thoracic aortas from multiorgan donors are suitable for obtaining resident angiogenic mesenchymal stromal cells," *Stem Cells*, vol. 25, no. 7, pp. 1627–1634, 2007.

[75] P. Campagnolo, D. Cesselli, A. Al Haj Zen et al., "Human adult vena saphena contains perivascular progenitor cells endowed with clonogenic and proangiogenic potential," *Circulation*, vol. 121, no. 15, pp. 1735–1745, 2010.

[76] V. Planat-Benard, J.-S. Silvestre, B. Cousin et al., "Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives," *Circulation*, vol. 109, no. 5, pp. 656–663, 2004.

[77] L. Zhou, J. Xia, X. Qiu et al., "In vitro evaluation of endothelial progenitor cells from adipose tissue as potential angiogenic cell sources for bladder angiogenesis," *PLoS ONE*, vol. 10, no. 2, Article ID e117644, 2015.

[78] S. Xue, H.-T. Zhang, P. Zhang et al., "Functional endothelial progenitor cells derived from adipose tissue show beneficial effect on cell therapy of traumatic brain injury," *Neuroscience Letters*, vol. 473, no. 3, pp. 186–191, 2010.

[79] X. Zhao, N. Wu, and L. Huang, "Endothelial progenitor cells and spleen: new insights in regeneration medicine," *Cytotherapy*, vol. 12, no. 1, pp. 7–16, 2010.

[80] M. J. Kiel, Ö. H. Yilmaz, T. Iwashita, O. H. Yilmaz, C. Terhorst, and S. J. Morrison, "SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells," *Cell*, vol. 121, no. 7, pp. 1109–1121, 2005.

[81] S. Wassmann, N. Werner, T. Czech, and G. Nickenig, "Improvement of endothelial function by systemic transfusion of vascular progenitor cells," *Circulation Research*, vol. 99, no. 8, pp. e74–e83, 2006.

[82] L. Werner, V. Deutsch, I. Barshack, H. Miller, G. Keren, and J. George, "Transfer of endothelial progenitor cells improves myocardial performance in rats with dilated cardiomyopathy induced following experimental myocarditis," *Journal of Molecular and Cellular Cardiology*, vol. 39, no. 4, pp. 691–697, 2005.

[83] T. Asahara, T. Takahashi, H. Masuda et al., "VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells," *The EMBO Journal*, vol. 18, no. 14, pp. 3964–3972, 1999.

[84] B. Heissig, K. Hattori, S. Dias et al., "Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of Kit-ligand," *Cell*, vol. 109, no. 5, pp. 625–637, 2002.

[85] A. Aicher, C. Heeschen, C. Mildner-Rihm et al., "Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells," *Nature Medicine*, vol. 9, no. 11, pp. 1370–1376, 2003.

[86] K.-I. Sasaki, C. Heeschen, A. Aicher et al., "*Ex vivo* pretreatment of bone marrow mononuclear cells with endothelial NO synthase enhancer AVE9488 enhances their functional activity for cell therapy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 39, pp. 14537–14541, 2006.

[87] D. J. Ceradini, A. R. Kulkarni, M. J. Callaghan et al., "Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1," *Nature Medicine*, vol. 10, no. 8, pp. 858–864, 2004.

[88] S. Massberg, I. Konrad, K. Schürzinger et al., "Platelets secrete stromal cell-derived factor 1α and recruit bone marrow-derived progenitor cells to arterial thrombi in vivo," *Journal of Experimental Medicine*, vol. 203, no. 5, pp. 1221–1233, 2006.

[89] K.-I. Hiasa, M. Ishibashi, K. Ohtani et al., "Gene transfer of stromal cell-derived factor-1α enhances ischemic vasculogenesis and angiogenesis via vascular endothelial growth factor/endothelial nitric oxide synthase-related pathway: next-generation chemokine therapy for therapeutic neovascularization," *Circulation*, vol. 109, no. 20, pp. 2454–2461, 2004.

[90] N. Urao, M. Okigaki, H. Yamada et al., "Erythropoietin-mobilized endothelial progenitors enhance reendothelialization

via Akt-endothelial nitric oxide synthase activation and prevent neointimal hyperplasia," *Circulation Research*, vol. 98, no. 11, pp. 1405–1413, 2006.

[91] D. H. Walter, K. Rittig, F. H. Bahlmann et al., "Statin therapy accelerates reendothelialization: a novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells," *Circulation*, vol. 105, no. 25, pp. 3017–3024, 2002.

[92] U. Laufs, N. Werner, A. Link et al., "Physical training increases endothelial progenitor cells, inhibits neointima formation, and enhances angiogenesis," *Circulation*, vol. 109, no. 2, pp. 220–226, 2004.

[93] L. V. D'Uscio, L. A. Smith, A. V. Santhanam, D. Richardson, K. A. Nath, and Z. S. Katusic, "Essential role of endothelial nitric oxide synthase in vascular effects of erythropoietin," *Hypertension*, vol. 49, no. 5, pp. 1142–1148, 2007.

[94] P. Foubert, J.-S. Silvestre, B. Souttou et al., "PSGL-1-mediated activation of EphB4 increases the proangiogenic potential of endothelial progenitor cells," *Journal of Clinical Investigation*, vol. 117, no. 6, pp. 1527–1537, 2007.

[95] E. Chavakis, A. Aicher, C. Heeschen et al., "Role of β2-integrins for homing and neovascularization capacity of endothelial progenitor cells," *Journal of Experimental Medicine*, vol. 201, no. 1, pp. 63–72, 2005.

[96] E. Chavakis, A. Hain, M. Vinci et al., "High-mobility group box 1 activates integrin-dependent homing of endothelial progenitor cells," *Circulation Research*, vol. 100, no. 2, pp. 204–212, 2007.

[97] C.-H. Yoon, J. Hur, I.-Y. Oh et al., "Intercellular adhesion molecule-1 is upregulated in ischemic muscle, which mediates trafficking of endothelial progenitor cells," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 26, no. 5, pp. 1066–1072, 2006.

[98] S.-P. Lee, S.-W. Youn, H.-J. Cho et al., "Integrin-linked kinase, a hypoxia-responsive molecule, controls postnatal vasculogenesis by recruitment of endothelial progenitor cells to ischemic tissue," *Circulation*, vol. 114, no. 2, pp. 150–159, 2006.

[99] C. Urbich, C. Heeschen, A. Aicher et al., "Cathepsin L is required for endothelial progenitor cell-induced neovascularization," *Nature Medicine*, vol. 11, no. 2, pp. 206–213, 2005.

[100] X. W. Cheng, M. Kuzuya, K. Nakamura et al., "Mechanisms underlying the impairment of ischemia-induced neovascularization in matrix metalloproteinase 2-deficient mice," *Circulation Research*, vol. 100, no. 6, pp. 904–913, 2007.

[101] J. Rehman, J. Li, C. M. Orschell, and K. L. March, "Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors," *Circulation*, vol. 107, no. 8, pp. 1164–1169, 2003.

[102] T. He, T. E. Peterson, and Z. S. Katusic, "Paracrine mitogenic effect of human endothelial progenitor cells: role of interleukin-8," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 289, no. 2, pp. H968–H972, 2005.

[103] G. P. Fadini, I. Baesso, M. Albiero, S. Sartore, C. Agostini, and A. Avogaro, "Technical notes on endothelial progenitor cells: ways to escape from the knowledge plateau," *Atherosclerosis*, vol. 197, no. 2, pp. 496–503, 2008.

[104] Z. Yang, M. W. von Ballmoos, D. Faessler et al., "Paracrine factors secreted by endothelial progenitor cells prevent oxidative stress-induced apoptosis of mature endothelial cells," *Atherosclerosis*, vol. 211, no. 1, pp. 103–109, 2010.

[105] M. Wyler von Ballmoos, Z. Yang, J. Völzmann, I. Baumgartner, C. Kalka, and S. Di Santo, "Endothelial progenitor cells induce a phenotype shift in differentiated endothelial cells towards PDGF/PDGFRβ axis-mediated angiogenesis," *PLoS ONE*, vol. 5, no. 11, article e14107, 2010.

[106] F. Meng, L. Liu, P. C. Chin, and S. R. D'Mello, "Akt is a downstream target of NF-κB," *The Journal of Biological Chemistry*, vol. 277, no. 33, pp. 29674–29680, 2002.

[107] A. Kebir, K. Harhouri, B. Guillet et al., "CD146 short isoform increases the proangiogenic potential of endothelial progenitor cells in vitro and in vivo," *Circulation Research*, vol. 107, no. 1, pp. 66–75, 2010.

[108] Y. Zhang, D. A. Ingram, M. P. Murphy et al., "Release of proinflammatory mediators and expression of proinflammatory adhesion molecules by endothelial progenitor cells," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 296, no. 5, pp. H1675–H1682, 2009.

[109] F. Dignat-George and C. M. Boulanger, "The many faces of endothelial microparticles," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 31, no. 1, pp. 27–33, 2011.

[110] V. Cantaluppi, S. Gatti, D. Medica et al., "Microvesicles derived from endothelial progenitor cells protect the kidney from ischemia-reperfusion injury by microRNA-dependent reprogramming of resident renal cells," *Kidney International*, vol. 82, no. 4, pp. 412–427, 2012.

[111] F. Jansen, X. Yang, M. Hoelscher et al., "Endothelial microparticle-mediated transfer of microRNA-126 promotes vascular endothelial cell repair via spred1 and is abrogated in glucose-damaged endothelial microparticles," *Circulation*, vol. 128, no. 18, pp. 2026–2038, 2013.

[112] N. Werner, J. Priller, U. Laufs et al., "Bone marrow-derived progenitor cells modulate vascular reendothelialization and neointimal formation: effect of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibition," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 22, no. 10, pp. 1567–1572, 2002.

[113] D. Kong, L. G. Melo, M. Gnecchi et al., "Cytokine-induced mobilization of circulating endothelial progenitor cells enhances repair of injured arteries," *Circulation*, vol. 110, no. 14, pp. 2039–2046, 2004.

[114] M. R. Schroeter, M. Leifheit, P. Sudholt et al., "Leptin enhances the recruitment of endothelial progenitor cells into neointimal lesions after vascular injury by promoting integrin-mediated adhesion," *Circulation Research*, vol. 103, no. 5, pp. 536–544, 2008.

[115] K. Strehlow, N. Werner, J. Berweiler et al., "Estrogen increases bone marrow-derived endothelial progenitor cell production and diminishes neointima formation," *Circulation*, vol. 107, no. 24, pp. 3059–3065, 2003.

[116] Y. Yoshida, N. Fukuda, A. Maeshima et al., "Treatment with valsartan stimulates endothelial progenitor cells and renal label-retaining cells in hypertensive rats," *Journal of Hypertension*, vol. 29, no. 1, pp. 91–101, 2011.

[117] F. Cacciatore, G. Bruzzese, D. F. Vitale et al., "Effects of ACE inhibition on circulating endothelial progenitor cells, vascular damage, and oxidative stress in hypertensive patients," *European Journal of Clinical Pharmacology*, vol. 67, no. 9, pp. 877–883, 2011.

[118] D. S. Jacoby and D. J. Rader, "Renin-angiotensin system and atherothrombotic disease: from genes to treatment," *Archives of Internal Medicine*, vol. 163, no. 10, pp. 1155–1164, 2003.

[119] T. Sugiura, T. Kondo, Y. Kureishi-Bando et al., "Nifedipine improves endothelial function: role of endothelial progenitor cells," *Hypertension*, vol. 52, no. 3, pp. 491–498, 2008.

[120] J. Llevadot, S. Murasawa, Y. Kureishi et al., "HMG-CoA reductase inhibitor mobilizes bone marrow-derived endothelial progenitor cells," *The Journal of Clinical Investigation*, vol. 108, no. 3, pp. 399–405, 2001.

[121] M. Vasa, S. Fichtlscherer, K. Adler et al., "Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease," *Circulation*, vol. 103, no. 24, pp. 2885–2890, 2001.

[122] A. M. Leone, S. Rutella, M. B. Giannico et al., "Effect of intensive vs standard statin therapy on endothelial progenitor cells and left ventricular function in patients with acute myocardial infarction: Statins for regeneration after acute myocardial infarction and PCI (STRAP) trial," *International Journal of Cardiology*, vol. 130, no. 3, pp. 457–462, 2008.

[123] C. Schmidt-Lucke, S. Fichtlscherer, A. Aicher et al., "Quantification of circulating endothelial progenitor cells using the modified ISHAGE protocol," *PLoS ONE*, vol. 5, no. 11, Article ID e13790, 2010.

[124] C. Spadaccio, F. Pollari, A. Casacalenda et al., "Atorvastatin increases the number of endothelial progenitor cells after cardiac surgery: a randomized control study," *Journal of Cardiovascular Pharmacology*, vol. 55, no. 1, pp. 30–38, 2010.

[125] C. Yost, M. Torres, J. R. Miller, E. Huang, D. Kimelman, and R. T. Moon, "The axis-inducing activity, stability, and subcellular distribution of β-catenin is regulated in Xenopus embryos by glycogen synthase kinase 3," *Genes and Development*, vol. 10, no. 12, pp. 1443–1454, 1996.

[126] A. Aicher, O. Kollet, C. Heeschen et al., "The wnt antagonist dickkopf-1 mobilizes vasculogenic progenitor cells via activation of the bone marrow endosteal stem cell niche," *Circulation Research*, vol. 103, no. 8, pp. 796–803, 2008.

[127] Y. P. R. Jarajapu and M. B. Grant, "The promise of cell-based therapies for diabetic complications challenges and solutions," *Circulation Research*, vol. 106, no. 5, pp. 854–869, 2010.

[128] B. Hibbert, J. R. Lavoie, X. Ma et al., "Glycogen synthase kinase-3β inhibition augments diabetic endothelial progenitor cell abundance and functionality via cathepsin B: a novel therapeutic opportunity for arterial repair," *Diabetes*, vol. 63, no. 4, pp. 1410–1421, 2014.

[129] C. Herencia, J. M. Martínez-Moreno, C. Herrera et al., "Nuclear translocation of β-catenin during mesenchymal stem cells differentiation into hepatocytes is associated with a tumoral phenotype," *PLoS ONE*, vol. 7, no. 4, Article ID e34656, 2012.

[130] M. A. Jordan, R. J. Toso, D. Thrower, and L. Wilson, "Mechanism of mitotic block and inhibition of cell proliferation by taxol at low concentrations," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 20, pp. 9552–9556, 1993.

[131] D. I. Axel, W. Kunert, C. Göggelmann et al., "Paclitaxel inhibits arterial smooth muscle cell proliferation and migration in vitro and in vivo using local drug delivery," *Circulation*, vol. 96, no. 2, pp. 636–645, 1997.

[132] R. Gallo, A. Padurean, T. Jayaraman et al., "Inhibition of intimal thickening after balloon angioplasty in porcine coronary arteries by targeting regulators of the cell cycle," *Circulation*, vol. 99, no. 16, pp. 2164–2170, 1999.

[133] G. J. Padfield, O. Tura, M. L. A. Haeck et al., "Circulating endothelial progenitor cells are not affected by acute systemic inflammation," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 298, no. 6, pp. H2054–H2061, 2010.

[134] J. Aoki, P. W. Serruys, H. van Beusekom et al., "Endothelial progenitor cell capture by stents coated with antibody against CD34: the HEALING-FIM (Healthy Endothelial Accelerated Lining Inhibits Neointimal Growth-First in Man) registry," *Journal of the American College of Cardiology*, vol. 45, no. 10, pp. 1574–1579, 2005.

[135] M. A. M. Beijk, M. Klomp, N. J. W. Verouden et al., "Genous™ endothelial progenitor cell capturing stent vs. the Taxus Liberté stent in patients with de novo coronary lesions with a high-risk of coronary restenosis: a randomized, single-centre, pilot study," *European Heart Journal*, vol. 31, no. 9, pp. 1055–1064, 2010.

[136] H. J. Duckers, S. Silber, R. de Winter et al., "Circulating endothelial progenitor cells predict angiographic and intravascular ultrasound outcome following percutaneous coronary interventions in the HEALING-II trial: evaluation of an endothelial progenitor cell capturing stent," *EuroIntervention*, vol. 3, no. 1, pp. 67–75, 2007.

[137] W. K. den Dekker, J. H. Houtgraaf, S. M. Rowland et al., "Efficiency of statin treatment on EPC recruitment depends on baseline EPC titer and does not improve angiographic outcome in coronary artery disease patients treated with the Genous stent," *Cell Transplant*, vol. 23, no. 12, pp. 1525–1535, 2014.

[138] T. Pereira-da-Silva, L. Bernardes, D. Cacela et al., "Safety and effectiveness of the genous endothelial progenitor cell-capture stent: follow-up to 5 years," *Journal of Invasive Cardiology*, vol. 25, no. 12, pp. 666–669, 2013.

[139] J. Wöhrle, R. Birkemeyer, S. Markovic et al., "Prospective randomised trial evaluating a paclitaxel-coated balloon in patients treated with endothelial progenitor cell capturing stents for de novo coronary artery disease," *Heart*, vol. 97, no. 16, pp. 1338–1342, 2011.

[140] J. F. Granada, S. Inami, M. S. Aboodi et al., "Development of a novel prohealing stent designed to deliver sirolimus from a biodegradable abluminal matrix," *Circulation: Cardiovascular Interventions*, vol. 3, no. 3, pp. 257–266, 2010.

[141] M. Haude, S. W. L. Lee, S. G. Worthley et al., "The REMEDEE trial: a randomized comparison of a combination sirolimus-eluting endothelial progenitor cell capture stent with a paclitaxel-eluting stent," *JACC: Cardiovascular Interventions*, vol. 6, no. 4, pp. 334–343, 2013.

[142] B. E. Strauer, M. Brehm, T. Zeus et al., "Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans," *Circulation*, vol. 106, no. 15, pp. 1913–1918, 2002.

[143] B. Assmus, V. Schächinger, C. Teupe et al., "Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI)," *Circulation*, vol. 106, no. 24, pp. 3009–3017, 2002.

[144] G. P. Meyer, K. C. Wollert, and H. Drexler, "Stem cell therapy: a new perspective in the treatment of patients with acute myocardial infarction," *European Journal of Medical Research*, vol. 11, no. 10, pp. 439–446, 2006.

[145] C. Stamm, B. Westphal, H.-D. Kleine et al., "Autologous bone-marrow stem-cell transplantation for myocardial regeneration," *The Lancet*, vol. 361, no. 9351, pp. 45–46, 2003.

[146] V. Schächinger, S. Erbs, A. Elsässer et al., "Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction," *New England Journal of Medicine*, vol. 355, no. 12, pp. 1210–1221, 2006.

[147] J. H. Traverse, T. D. Henry, C. J. Pepine et al., "Effect of the use and timing of bone marrow mononuclear cell delivery on left ventricular function after acute myocardial infarction: the TIME randomized trial," *The Journal of the American Medical Association*, vol. 308, no. 22, pp. 2380–2389, 2012.

[148] D. Sürder, R. Manka, V. Lo Cicero et al., "Intracoronary injection of bone marrow-derived mononuclear cells early or late after acute myocardial infarction: effects on global left ventricular function," *Circulation*, vol. 127, no. 19, pp. 1968–1979, 2013.

[149] L. R. Gao, X. T. Pei, Q. A. Ding et al., "A critical challenge: dosage-related efficacy and acute complication intracoronary injection of autologous bone marrow mesenchymal stem cells in acute myocardial infarction," *International Journal of Cardiology*, vol. 168, no. 4, pp. 3191–3199, 2013.

Chapter 7

Curriculum vitae

POST-SECONDARY EDUCATION

2015 – Present	Doctor in Medicine (MD) – University of Ottawa Doctorate of Philosophy (PhD) – Cellular and Molecular Medicine – University of Ottawa
2010 - 2015	Bachelor of Science – Biochemistry (Honours) – University of Manitoba Gold Medal – Highest Academic Standing Graduated with First Class Honours

PEER-REVIEWED PUBLICATIONS

Published (*shared first authorship)

1. Mathew R, Di Santo P, **Jung RG**, Marbach J, Hutson J, Simard T, Ramirez RF, Harnett DT, Merdad A, Almufleh A, Weng W, Abdel-Razek O, Fernando S, Kyeremanteng, K, Bernick J, Wells G, Chan V, Froeschl M, Labinaz, M, Le May M, Russo J, Hibbert B. Comparison of milrinone vs dobutamine in the treatment of cardiogenic shock. (**2021**). *New England Journal of Medicine*.

2. Di Santo P, Mathew R, **Jung RG**, Simard T, Skanes S, Mao B, Ramirez FD, Marbach JA, Abdel-Razek O, Motazedian P, Parlow S, Boczar K, D'Egidio G, Hawken S, Dick A, So DY, Glover C, Russo JJ, McGuinty C, Hibbert B. The paradoxical relationship between beta-blocker use and cardiogenic shock. (**2021**). *Critical Care.*

3. Clifford CR, **Jung RG**, Hibbert B, Chong AY, Tanguay JF, Lordkipanidze M, So D. Dual antiplatelet therapy (PEGASUS) vs. dual pathway (COMPASS): A head-to-head *in vitro* comparison. **(2021)**. *Platelets*.

4. **Jung RG**, Di Santo P, Clifford C, Prosperi-Porta G, Skanes S, Hung A, Visintini S, Parlow S, Ramirez FD, Simard T, Hibbert B. Methodological quality of COVID-19 clinical research: a systematic review and matched cohort analysis. (**2021**). *Nature Communications*

5. **Jung RG**, Simard T, Kovach C, Flint K, Don C, Di Santo P, Adamo M, Branca L, Valentini F, Benito-Gonzalez T, Vazquez FF, Estevez-Loureiro R, Berardini A, Conti N, Rapezzi C, Biagini E, Parlow S, Shorr R, Levi A, Manovel A, Cardenal-Piris R, Diaz JF, Shuvy M, Haberman D, Alessandra S, Marini C, Bargagna M, Schiavi D, Paolo D, Buzzatti N, Markovic S, Chan V, Hynes M, Mesana T, Labinaz M, Markovic S, Pappalardo F, Taramasso M, Hibbert B. Transcatheter mitral-valve repair in cardiogenic shock and severe mitral regurgitation: a patient-level multi-center analysis. (**2021**). *JACC: Cardiovascular Interventions*.

6. Di Santo P, Simard T, Wells GA, **Jung RG**, Ramirez FD, Boland P, Marbach JA, Parlow S, Kyeremanteng K, Coyle D, Fergusson D, Russo JJ, Chong AY, So DY, Dick A, Glover C, Labinaz M, Hibbert B, Le May M. Transradial versus transfemoral access for percutaneous coronary intervention in ST-elevation myocardial infarction: a systematic review and meta-analysis. (**2021**). *Circulation: Cardiovascular Interventions*.

7. Karunakaran D, Nguyen MA, Geoffrion M, Vreeken D, Lister Z, Cheng HS, Otte N, Essebier P, Wyatt H, Kandiah JW, **Jung R**, Alenghat FJ, Mompeon A, Lee R, Pan C, Gordon E, Rasheed A, Lusis AJ, Liu P, Matic LP, Hedin U, Fish JE, Guo L, Kolodgie F, Virmani R, Rayner KJ.

Therapeutic knockdown of RIPK1 gene expression reduces NF-κB inflammation in macrophages and prevents atherosclerotic lesion development. (**2021**). *Circulation*.

8. **Jung RG***, Sarathy K, Simard T, Parlow S, Di Santo P, Moreland R, Jung Y, Abdel-Razek O, Boland P, Russo JJ, Chong AY, So D, Froeschl M, Dick A, Glover C, Labinaz M, Le May M, Hibbert B. Physician prediction of one-year mortality in the cardiac catheterization laboratory: comparisons to a validated risk score. **(2021)**. *Coronary Artery Disease*.

9. Rasheed A, Shawky S, Tsai R, **Jung RG**, Simard T, Saikali M, Hibbert B, Rayner KJ, Cummins CL. The secretome of liver X receptor agonist treated early outgrowth cells decreases atherosclerosis in Ldlr-/- mice. **(2020)**. *Stem Cell Translational Medicine*.

10. Simard T, Motazedian P, Majeed K, Sarathy K, **Jung RG**, Ramirez FD, Feder J, Di Santo P, Parlow S, Short S, Labinaz A, Schultz C, So D, Chong AY, Le May M, Hibbert B. Contrast-free optical coherence tomography: systematic evaluation of non-contrast media for intravascular assessment. **(2020)**. *PLoS ONE.*

11. **Jung RG***, Simard T, Di Santo P, Ramirez FD, Labinaz A, Gaudet C, Motazedian P, Parlow S, Joseph J, Moreland R, Marbach JA, Boland P, Promislow S, Russo JJ, Chong AY, So D, Labinaz M, Le May M, Hibbert B. Performance of plasma adenosine as a biomarker for predicting cardiovascular risk. (**2020**). *Clinical and Translational Science*.

12. **Jung RG**, Di Santo P, Hibbert B. Commentary: geographic variation in the statin trials – underrepresentation of Asian populations. (**2020**). *International Journal of Cardiology*.

13. Marbach JA, Almufleh A, Di Santo P, Simard T, **Jung RG**, Diemer G, West FM, Millington SJ, Mathew R, Le May M, Hibbert B. A shifting paradigm – the role of focused cardiac ultrasound in bedside patient assessment. (**2020**). *Chest*.

14. **Jung RG**, Simard T, Di Santo P, Dhaliwal S, Sypkes C, Duchez AC, Moreland R, Taylor K, Parlow S, Visintini S, Labinaz A, Sarathy K, Bernick J, Abdel-Razek O, Harnett DT, Boland P, Joseph J, Ramirez FD, Hibbert B. Plasma plasminogen activator inhibitor-1 as a biomarker of unplanned revascularization and major adverse cardiac events in patients undergoing coronary angiography. (**2020**). *Thrombosis Research*.

15. **Jung RG**, Parlow S, Simard T, Chen C, Ghataura H, Kishore A, Parera A, Moreland R, Hughes I, Tavella R, Hibbert B, Beltrame J, Singh K. Clinical features, sex differences and outcomes of myocardial infarction with nonobstructive coronary arteries (MINOCA): a multi-centre, international, registry analysis. **(2020)**. *Coronary Artery Disease.*

16. Ramirez FD, **Jung RG**, Motazedian P, Perry-Nguyen D, Di Santo P, MacDonald Z, Clancy A, Labinaz A, Promislow S, Simard T, Provencher S, Bonnet S, Graham I, Wells G, Hibbert B. Journal initiatives to enhance preclinical research: Analyses of Stroke, Nature Medicine, Science Translational Medicine. **(2020)**. *Stroke.*

17. Labinaz A, Marbach JA, **Jung RG**, Moreland R, Motazedian P, Di Santo P, Clancy AA, MacDonald Z, Simard T, Hibbert B. Female authorship in preclinical cardiovascular research: temporal trends and influence on experimental design. (**2019**). *JACC: Basic to Translational Science.*

18. **Jung RG***, Simard T*, Labinaz A, Faraz MA, Ramirez FD, Di Santo P, Perry-Nguyen D, Pitcher I, Motazedian P, Gaudet C, Rochman R, Marbach J, Boland P, Sarathy K, Alghofaili S, Russo JJ, Couture E, Promislow S, Beanlands RS, Hibbert B. Circulating adenosine as a biomarker of cardiovascular outcomes: technical and biological considerations. **(2019).** *Journal of American Heart Association.*

19. Marbach JA, Almufleh A, Di Santo P, **Jung RG**, Simard T, McInnes MD, Salameh JP, McGrath TA, Millington SJ, Diemer G, West FM, Domecq MC, Hibbert B. The diagnostic impact of focused cardiac ultrasound in addition to physical exam for valvular heart disease and left ventricular dysfunction: a systematic review & meta-analysis. **(2019)**. *Annals of Internal Medicine.*

20. **Jung RG**, Simard T, Di Santo P, Labinaz A, Moreland R, Duchez AC, Majeed K, Motazedian P, Rochman R, Jung Y, Hibbert B. Performance of plasminogen activator inhibitor-1 as a biomarker in patients undergoing coronary angiography: Analytical and biological considerations. (**2019**). *Diabetes and Vascular Disease Research.*

21. **Jung RG***, Perry-Nguyen D***,** Labinaz A, Duchez AC, Dewidar O, Simard T, Karunakaran D, Majeed K, Sarathy K, Li R, Ramirez FD, Di Santo P, Rochman R, So D, Foin N, Hibbert B. Evaluation of an *in vitro* coronary stent thrombosis model for preclinical assessment. (**2019**). *Platelets.*

22. **Jung RG***, Simard T*, Labinaz A, Faraz MA, Ramirez FD, Di Santo P, Pitcher I, Motazedian P, Gaudet C, Rochman R, Marbach J, Boland P, Sarathy K, Alghofaili S, Russo JJ, Couture E, Beanlands RS, Hibbert B. Adenosine as a marker and mediator of cardiovascular homeostasis: a translational perspective. **(2018).** *CHDDT.*

23. **Jung RG***, Motazedian P*, Ramirez FD, Simard T, Di Santo P, Visintini S, Faraz MA, Labinaz A, Jung Y, Hibbert B. Association between plasminogen activator inhibitor-1 and cardiovascular events: a systematic review and meta-analysis. **(2018).** *Thrombosis Journal.*

24. Di Santo P**,** Harnett DT, Simard T, Ramirez FD, Pourdjabbar A, Yousef A, Moreland R, Bernick J, Wells GA, Dick A, Le May M, Labinaz M, So D, Motazedian P, **Jung RG**, Chandrashekar J, Mehran R, Chong AY, Hibbert B. Photoplethysmography using a smartphone application for assessment of ulnar artery patency: a randomized clinical trial. **(2018)**. *CMAJ.* 190(13):E380-E388

25. **Jung RG***, Simard T*, Labinaz A, Ramirez FD, Di Santo P, Motazedian P, Rochman R, Gaudet C, Faraz MA, Beanlands RSB, Hibbert B. Role of plasminogen activator inhibitor-1 in coronary pathophysiology. **(2018).** *Thrombosis Research*.164:54-62.

26. Di Santo P, Motazedian P, **Jung RG**, Simard T, Ramirez FD, Chong AY, Glover C, Hibbert B, Dwivedi G. Evaluation of cobalt and chromium levels following implantation of coronary stents: a pilot study. **(2017).** *Heart, Lung, Circulation Journal.*

27. Ramirez FD, Motazedian P, **Jung RG**, Di Santo P, MacDonald ZD, Moreland R, Simard T, Clancy AA, Russo JJ, Welch VA, Wells GA, Hibbert B. Methodological Rigor in Preclinical Cardiovascular Studies: Targets to Enhance Reproducibility and Promote Research Translation. **(2017)**. *Circulation Research*. 120(12):1916-1926.

28. Ramirez FD, Motazedian P, **Jung RG**, Di Santo P, MacDonald Z, Simard T, Hibbert B. Sex bias is Increasingly Prevalent in Preclinical Cardiovascular Research: Implications for Translational Medicine and Health Equity for Women. **(2017)**. *Circulation. 135(6):625-626.*

29. **Jung RG***, Simard T*, Motazedian P, Di Santo P, Ramirez FD, Russo J, Labinaz A, Yousef A, Anantharam B, Pourdjabbar A, Hibbert B. Progenitor cells for arterial repair: incremental advancements towards therapeutic reality. **(2017)**. *Stem Cells International.* 2017:ID 8270498

30. **Jung RG**, Radko S, Pelka P. The Dual Nature of Nek9 in Adenovirus Replication. **(2015).** *J Virology, 90*: 1931-1943. doi:10.1128/JVI.02392-15. Article selected as Editor's Choice and cover of the issue.

31. Radko S, **Jung RG**, Olanubi O, Pelka P. Effects of Adenovirus Type 5 E1A Isoforms on Viral Replication in Arrested Human Cells. **(2015).** *PLoS One, 10*: e0140124. doi:10.1371/journal.pone.0140124

32. Radko S, Koleva M, James KM, **Jung RG**, Mymryk JS, Pelka P. Adenovirus E1A targets the DREF nuclear factor to regulate virus gene expression, DNA replication, and growth. **(2014).** *J Virology, 88*: 13469-13481. doi:10.1128/JVI.02538-14

In Preparation/Review (*shared first authorship)

1. **Jung RG***, Simard T*, Lehenbauer K, Piayda K, Pracon R, …, Sievert H, Main ML, Hibbert B Holmes DR, Alkhouli M. Prediction of device-related thrombus following percutaneous left atrial appendage occlusion. (**2021**). *Under review at JACC.*

2. **Jung RG**, Duchez AC, Simard T, Dhaliwal S, Gillmore T, Di Santo P, Labinaz A, Ramirez FD, Rasheed A, Robichaud S, Ouimet M, Short S, Clifford C, Xiao F, Lordkipanidze M, Burger D, Gadde S, Rayner K, Hibbert B. Plasminogen activator inhibitor-1 platelet-derived extracellular vesicles predicts major adverse cardiac events and promotes the pro-inflammatory smooth muscle cell phenotype. (**2021**). *Under review at Circulation.*

3. Verreault-Julien L, Bhatt DL, **Jung RG**, Di Santo P, Simard T, Avram R, Hibbert B. Predictors of angina resolution after percutaneous coronary intervention in stable coronary artery disease. (**2021**) *Under review at JACC: Cardiovascular Interventions*.

4. **Jung RG***, Simard T, Di Santo P, Harnett DT, Abdel-Razek O, Ramirez FD, Motazedian P, Parlow S, Moreland R, Labinaz A, Marbach J, Poulin A, Levi A, Majeed K, Boland P, Sarathy K, Promislow S, Russo JJ, Chong AY, So D, Froeschl M, Dick A, Glover C, Labinaz M, Le May M, Hibbert B. Modifiable risk factors and residual risk following coronary revascularization: insights from a regionalized dedicated follow-up clinic. (**2021**). *Submitted*.

5. Simard T, Motazedian P, Dhaliwal S, Di Santo P, **Jung RG**, Ramirez FD, Labinaz A, Short S, Parlow S, Joseph J, Rasheed A, Rockley M, Marbach J, Domecq MC, Russo JJ, Chong AY, Beanlands RS, Hibbert B. Revisiting the evidence for dipyridamole in reducing restenosis: a systematic review and meta-analysis. (**2021**). *Submitted*.

6. **Jung RG,** Abdel-Razek O, Di Santo P, Gillmore T, Sypkes C, Ramirez FD, Simard T, Hibbert B. Atrial fibrillation in the setting of atherosclerotic cardiovascular disease is associated with major adverse cardiovascular events. (**2021**). *In progress*

7. **Jung RG**, Di Santo P, Mathew R, Abdel-Razek O, Parlow S, Simard T, Marbach JA, Gillmore T, Mao B, Bernick J, Theriault-Lauzier P, Fu A, Lau L, Motazedian P, Russo JJ, Chong AY, Le May M, Labinaz M, Hibbert B. Implications of myocardial infarction on management and outcome in cardiogenic shock: a sub-analysis from the CAPITAL DOREMI study. (**2021**). *Submitted to EHJACC*.

8. **Jung RG**, Dhaliwal S, Makwana D, Moreland R, Di Santo P, Clifford C, Prosperi-Porta G, Skanes S, Hung A, Visintini S, Parlow S, Gillmore T, Ramirez FD, Simard T, Hibbert B. Female authorship in COVID-19 clinical research and methodological quality. (**2021**). *In progress*.

SCIENTIFIC MEETINGS

1. Mathew R, Di Santo P, Marbach J, Hutson J, Simard T, **Jung R,** Merdad A, Almufleh A, Weng W, Abdel-Razek O, Fernando S, Kyeremanteng K, Bernick J, Wells G, Froeschl M, Labinaz M, Le May M, Russo JJ, and Hibbert B. Capital Do-Re-Mi: A Randomized Trial of Dobutamine Compared to Milrinone in Cardiogenic Shock. American Heart Association: Scientific Sessions Virtual Meeting, Chicago, United States. *Virtual presentation*.

2. **Jung RG**, Simard T, Kovach C, Flint K, Don C, Di Santo P, Adamo M, Branca L, Valentini F, Benito-Gonzalez T, Vazquez FF, Estevez-Loureiro R, Berardini A, Conti N, Rapezzi C, Biagini E, Parlow S, Shorr R, Levi A, Manovel A, Cardenal-Piris R, Diaz JF, Shuvy M, Haberman D, Alessandra S, Marini C, Bargagna M, Schiavi D, Paolo D, Buzzatti N, Markovic S, Chan V, Hynes M, Mesana T, Labinaz M, Markovic S, Pappalardo F, Taramasso M, Hibbert B. Transcatheter mitral-valve repair in cardiogenic shock and severe mitral regurgitation: a patient-level multi-center analysis. (**2020**). Transcatheter Cardiovascular Therapeutics (TCT) Connect, United States. *Oral abstract*.

3. **Jung RG (presenting author)**, Simard T, Di Santo P, Duchez AC, Labinaz A, Parlow S, Yang J, Dhaliwal S, Ramirez FD, Xiao F, Lordkipanidze M, Burger D, Hibbert B. Discovery of plasminogen activator inhibitor-1 platelet -derived extracellular vesicles to predict major adverse cardiac events. (**2020**). American Heart Association: Scientific Sessions Virtual Meeting, Chicago, United States. *Virtual presentation*.

4. **Jung RG**, Simard T, Dhaliwal S, Di Santo P, Parlow S, Abdel-Razek O, Rizk M, Joseph J, Sypkes C, Moreland R, Duchez AC, Burger D, Hibbert B. Discovery and validation of plasminogen activator inhibitor-1 platelet-derived extracellular vesicles to predict major adverse cardiac events. (**2020**). Transcatheter Cardiovascular Therapeutics Asia-Pacific (TCTAP) Virtual Meeting, Seoul, South Korea. *Moderated poster*.

5. Clifford C, **Jung RG**, Hibbert B, So D. Dual antiplatelet vs. dual pathway strategies: an in vitro mechanistic study. (**2020**) American College of Cardiology (ACC), Chicago, United States.

6. **Jung RG (presenting author),** Parlow S, Simard T, Chen C, Ghataura H, Kishore A, Parera A, Moreland R, Hughes I, Tavella R, Hibbert B, Beltrame J, Singh K. Clinical Features, Sex Differences and Outcomes of Myocardial Infarction With Non-Obstructive Coronary Arteries (MINOCA): A Multi-Center, International, Registry Analysis. **(2019)**. Transcatheter Cardiovascular Therapeutics (TCT), San Francisco, United States. *Moderated poster.*

7. Ghataura H, Chen C, Kishore A, Perera A, Simard T, **Jung R**, Parlow S, Hughes I, Hibbert B, Singh K. Sex-Based Differences in Net Adverse Cardiovascular Events (NACE) Among MINOCA Patients. Heart, Lung and Circulation. **(2019)**.

8. Singh K, Ghataura H, Chen C, Kishore A, Perera A, **Jung R**, Parlow S, Hibbert B, Simard T. Incidence, Clinical Characteristics and In-Hospital Outcomes of MINOCA Patients: A Combined Analysis from 2 Large Registry Datasets. Heart, Lung and Circulation. **(2019)**.

9. **Jung RG (presenting author)**, Simard T, Labinaz A, Ramirez FD, Di Santo P, Pitcher I, Motazedian P, Rochman R, Moreland R, Marbach J, Boland P, Sarathy K, Alghofaili S, Russo J, Couture E, Froeschl M, Dick A, Hibbert B. Impact of general cardiology follow up on optimal cardiovascular risk factor management post-revascularization: outcomes in a nationalized healthcare system. **(2018)**. Transcatheter Cardiovascular Therapeutics (TCT), San Diego, California, United States. *Moderated poster.*

10. Simard T, **Jung RG**, Di Santo P, Ramirez FD, Labinaz A, Pitcher I, Motazedian P, Rochman R, Moreland R, Marbach J, Boland P, Sarathy K, Alghofaili S, Russo J, Couture E, Dick A, Froeschl

M, Le May M, Hibbert B. The impact of weight loss on cardiovascular outcomes following percutaneous coronary revascularization - a contemporary, real world experience. **(2018).** Transcatheter Cardiovascular Therapeutics (TCT), San Diego, California, United States. *Moderated poster.*

11. Sarathy K, **Jung RG**, Labinaz A, Rochman R, Pitcher I, Moreland R, Motazedian P, Ramirez FD, Di Santo P, Marbach J, Boland P, Simard T, Alghofaili S, Russo J, Couture E, Labinaz M, Glover C, Hibbert B. The impact of weight loss on cardiovascular outcomes following percutaneous coronary revascularization - a contemporary, real world experience. **(2018).** Transcatheter Cardiovascular Therapeutics (TCT), San Diego, California, United States. *Moderated poster.*

12. Simard T, Di Santo P, Ramirez FD, **Jung RG**, Labinaz A, Pitcher I, Motazedian P, Rochman R, Moreland R, Marbach J, Boland P, Sarathy K, Alghofaili S, Russo J, Couture E, So D, Chong AY, Le May M, Hibbert B. Achieving target LDL following percutaneous coronary revascularization - contemporary results in a nationalized health care system. **(2018).** Transcatheter Cardiovascular Therapeutics (TCT), San Diego, California, United States. *Moderated poster.*

13. Di Santo P**,** Harnett DT, Simard T, Ramirez FD, Pourdjabbar A, Yousef A, Moreland R, Bernick J, Wells GA, Dick A, Le May M, Labinaz M, So DY, Motazedian P, **Jung RG**, Chong AY, Hibbert B. **(2016).** Photoplethysmography using a smartphone application for assessment of ulnar artery patency prior to transradial angiography: a randomized clinical trial. *American Heart Association: Scientific Sessions. Poster Presentation. New Orleans, Louisiana, USA.*

14. Radko S, Koleva M, **Jung RG,** Pelka P. **(2015).** The dual nature of a cellular protein DREF in adenovirus life cycle: a facilitator and restrictor of viral growth. *Canadian Student Health Research Forum. Poster Presentation. Winnipeg, Manitoba, Canada.*

GRANTS

2018 University of Ottawa Heart Institute Academic Medical Organization Alternate Funding Plans: Innovation Competition (PI: Dr. Benjamin Hibbert) - $50,000 for 2 years

AWARDS AND ACCOMPLISHMENTS

2020 CMA COVID-19 Support

2017 - 2020 Vanier CIHR Canada Graduate Scholarship

2017 Frederick Banting and Charles Best Canada Graduate Scholarship
- Declined in favour of Vanier CGS

2017 Ontario Graduate Scholarship
- Declined in favour of Vanier CGS

2017 – 2020 University of Ottawa Excellence Scholarship

2015 – 2022 University of Ottawa MD/PhD Scholarship

2015 University of Manitoba Gold Medal for Bachelor of Science Honours
- Awarded for highest standing academically amongst honours students

2015 Rhodes Scholarship Regional Finalist

2014 Winnipeg Jets True North Foundation Grant
- First annual CancerTalk Symposium and high school summer camp at the University of Manitoba

2014	Edna Hollier Award - Highest standing amongst 3rd year honours student in biochemistry
2012 – 2014	NSERC Undergraduate Summer Research Award
2012	Oscar Vong Memorial Scholarship - Highest standing in 2nd year microbiology
2012	Hugh J. Anderson Scholarship in Chemistry - Highest standing in 2nd year chemistry
2012	Archbishop Leonard V. Wall Scholarship in Catholic Studies - Highest standing in “Introduction to Catholic Studies”
2012	Canadian Society of Chemistry Medal - Highest standing in 2nd year chemistry
2011	Dr. & Mrs. Michael Trainor Scholarship - Highest standing at St. Paul’s College
2011	William Briskie Memorial Scholarship in English and Economics - Highest standing in economics at St. Paul’s College
2011	Isbister Scholarship in University 1 - Top 11 student in first year
2010 – 2015	Dean’s Honour List
2010	Advanced Early Admission Scholarship
2010	St. Paul’s College Alumni/Friends Association Entrance Scholarship

EXTRACURRICULAR ACTIVITIES AND CONTRIBUTIONS TO THE UNIVERSITY

University of Ottawa Heart Institute Trainee Committee (2018-2019)

- **Co-Chair**

Internal Medicine Summer Camp (2017-2018)

- **Co-Founder** – collaborated with residents and physicians from all divisions of medicine to organize clinics, lunch-time talks, and workshops for Pre-Clerkship medical students at the University of Ottawa
- Discussion of expansion to other faculties across Ontario

Let's Talk Science (2013-2016)

- **Cancer Conference Coordinator (Let's Talk Cancer)** – **University of Ottawa** – organized presentations for high school students in Ontario and a conference for Grade 9-12 students for April 2016
- **Site Coordinator (2013 – 2015)** – **University of Manitoba -** organized workshops and trained student volunteers to empower students in science literacy
    - Managed over 200 volunteers and outreached to 25,000 students in two years
    - Organized a Let's Talk Science Cancer Symposium for Grade 11-12 students

VP Global Health Assistant (2015 – 2016)

- Attended Aesculapian Society's monthly meetings and coordinated global health initiatives with the VP Global Health Jr. at the University of Ottawa
- Organized a global health conference for September 2016

St. Paul's College Board of Governors (2011-2012)

- **Senior Stick** – representing the students' needs and concerns for the College's Board of Governors
- Chair general and student association meetings to organize several socials and expand the role of the student committee

JOURNAL REVIEWER

Thrombosis Research (2020 -)
American Journal of Industrial Medicine (2019 -)
Platelets (2019 -)
University of Ottawa Journal of Medicine (2015-2016)

MEMBERSHIPS AND AFFILIATIONS

Canadian Medical Association (#641454)
Ontario Medical Association (#1301332)
American Association for the Advancement of Science
Canadian Cardiovascular Society (#138759)
International Society on Thrombosis and Haemostasis